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Original Article

Binding of Novobiocin Paves the Way for Inhibition of DEAH-box Helicase 8

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ABSTRACT

Introduction: DEAH-box helicase 8 (DHX8) is a crucial DEAH-box RNA helicase involved in splicing and required for the release of mature mRNA from the splice. Here, we report the interaction study of human DHX8 and four test compounds namely etoposide, netropsin, nogalamycin, and novobiocin. **Materials and Methods:** Molecular docking and fluorescence emission spectra techniques were employed to determine the binding and inhibitory effect of test compounds. **Results:** Our docking and fluorescence emission spectra results showed that DHX8 has a good binding preference for novobiocin among these four test compounds. Moreover, fluorescence emission spectra of DHX8 with novobiocin also revealed the 2.5 µM concentration is an effective novobiocin concentration to inhibit the activity of DHX8. **Conclusion:** These findings provide an in-depth understanding of the inhibition of DHX8 and contribute insights towards the development of novobiocin as a therapeutic molecule against the DHX8 in targeted diseases.

Keywords: DEAH-box helicase, DHX8, Novobiocin, Splicing, Therapeutics.

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INTRODUCTION

An essential DEAH-box RNA helicase involved in many fundamental biological processes, such as homeostasis and RNA splicing, is DHX8. There have been reports of DHX8 operating abnormally in several illnesses, including hepatocellular carcinoma with a poor prognosis. DEAH-box helicase 8 is a DEAH box polypeptide family member.[1] The encoded protein contains the DEAH (Asp-Glu-Ala-His) motif, which is found in all DEAH box proteins, and is thought to function as an ATP-dependent RNA helicase that regulates the release of spliced mRNAs from spliceosomes prior to nuclear export.[2,3] Multiple transcript variants result from alternative splicing. DHX8 is found in the nucleus and is activated by the presence of RNA.[4] Because this protein is a spliceosome component, it participates in pre-mRNA splicing. Furthermore, DHX8 is involved in the release of spliced mRNA, facilitating nuclear export. DHX8 prefers adenine-rich RNA, according to protein characterization. Following this binding, ATP hydrolysis occurs, resulting in ADP release.[4] DHX8 is made up of a highly variable N-terminal domain and a conserved C-terminal helicase domain, similar to other DEAH-box RNA helicases.[1] The latter contains two RecA domains, RecA1 and RecA2, which form the helicase core and contain up to 12 distinctive motifs involved in ATP binding and hydrolysis, RNA binding, and helicase activity.[5,6] In addition to the RecA domains, the DHX8 helicase domain contains Winged-Helix (WH), ratchet-like, and Oligonucleotide Binding (OB)-fold domains at the C-terminus.[7-10]

Several studies have been published on the activity and role of normal DHX8 as well as on its mutated forms.[11-13] These studies have also highlighted its association with important biological processes such as hematopoiesis and are linked to certain diseases. It has also been documented that this protein may be required for Human Immunodeficiency Virus type 1 replication (HIV-1). High Levels of DEAH-Box Helicases has been reported to correlate with poor prognosis of hepatocellular carcinoma.[14,15] All these findings together suggest that DHX8 can be considered as therapeutic target in DHX8 impairment associated diseases.

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Bioinformatics analysis is uniquely suited to research, describe, explore, and understand structural and relational aspects of targeted proteins with diseases using a theoretical paradigm and methodological tools.[16] Binding affinity studies are becoming an important tool for determining target protein binding affinity and relationships with ligands.[17,18] Such research has improved our understanding of drug targets and their effects, as well as suggested new drug targets, therapeutics, and therapeutic management approaches in severe diseases.[19]

Furthermore, binding affinity studies are making significant contributions to the field of systems pharmacology.

Current study was proposed to determine the interaction of human DHX8 protein with four test compounds namely etoposide, netropsin, nogalamycin, and novobiocin. Binding affinity was determined by molecular docking and Fluorescence Emission Spectra (FES) studies. Our docking and fluorescence emission spectra revealed that DHX8 has good binding preference for novobiocin among these four test compounds. Findings of the current study provided in-depth understanding of the inhibition of DHX8 and contribute insights towards the development of novobiocin as therapeutic molecule against the DHX8 in targeted diseases. We report that each biochemical representation of the DHX8 contains the brand nucleic acid ATPase, and Novobiocin inhibits hydrolysis activity at low concentrations. The findings presented here will further our understanding of the nucleic acid metabolic processes in the diseases studied.

MATERIALS AND METHODS

Retrieval and preparation of target protein DHX8 and test compounds

The 3D structure of target protein DHX8 (2.3 Angstrom) was retrieved from RCSB-PDB (ID: 6HYT) (Figure 1). Structure of the test compounds (Figures 2A-D) namely etoposide, netropsin, nogalamycin, and novobiocin were retrieved from PubChem database as .sdf files and their energy was minimized using LigPrep module of Maestro. All possible ionization states at pH 7.0 ± 2.0 were generated and minimized. Ligand molecules generated were docked into the active binding site of DHX8 in Standard/Extra Precision mode (SP/XP) using Glide.

Molecular docking

Molecular docking study was performed to establish interactions of test compounds with the target protein DEAH-box Helicase 8 (DHX8). Molecular docking study was carried out on the 3D structure of target protein retrieved from the provided link (as DHX8_model_01.pdb) using Maestro 10.1 program (Schrodinger Inc., USA). The target protein for the present study was first prepared using protein preparation wizard tool. Water molecules and all other undesirable residues were removed after preprocessing. It was then subjected to hydrogen bond optimization and energy minimization. Using 'Sitemap' tool of Maestro, most probable binding sites were identified, and the best binding site identified for the target protein was selected for further processing. The binding site was defined as grid box using receptor grid generation tool in Glide.

Fluorescence Emission Spectra based inhibition study of DHX8 by Novobiocin

Fluorescence spectra of native protein "DHX8" and DHX8-novobiocin complex were measured using a 1cm quartz cell in a JASCO 6300 spectrofluorometer. A slit width of 10nm was used for both excitation and emission. Human Recombinant Protein DHX8 (Boster Bio, Cat # PROTQ14562) spectra were obtained for the intrinsic tryptophan fluorescence assay. The binding of DHX8 to novobiocin (Sigma, CAS No.: 56677-21-5) was investigated using Fluorescence Emission Spectra. Samples were stimulated at 280 nm, and emission spectra were recorded from 300 to 500 nm.

Hydrolysis Assay

For further docking and fluorescence confirmation, we have done kinase profiling of DHX8 protein on different concentration (25, 50 and 100ng). We also tested kinetics (*Km*) value in the presence of inhibitor on RNA-dependent ATPase assay. By determining the formation of ^{32}P from [32P] ATP, the hydrolysis of ATP catalyzed by DHX8 protein was mixed with buffer.[20] The reaction was carried out for one hour at 37°C in the presence of proteins (25, 50, and 100ng). By keeping the reaction mixture of [32P] ATP and cold ATP (1 mM) in the ice, the reaction was stopped. One of each reaction mixture was spotted onto a polyethyleneimine-cellulose thin-layer strip, and chromatography was used for 30 min at room temperature in a suitable solution with 0.5 M LiCl and 1M formic acid. The strip was dried and exposed to hyper film to identify the ATP and Pi hot spots. Quantitation was performed using the Alpha Imager-EP/Image-J software (http://rsbweb.nih.gov/ij/).[21]

Inhibitor study on ATPase and kinetics

To investigate the effect of the ligands (Novobiocin) on hydrolysis, DNA was preincubated with DNA-interacting ligands (Novobiocin) for 20 min before adding the DHX8 protein. The experiments were carried out, and the percentage hydrolysis was calculated using Microsoft Excel 2010. In order to understand the mechanism of inhibitor (DNA interacting compound) on the degined assay. The ATPase assay for estimating the kinetic parameters of DHX8 was carried out with DNA-interacting ligands (Novobiocin). GraphPad Prism Software 5 was used to calculate the IC_{50} and kinetic parameter (*Km*).[10]

Data analysis

Using ImageJ 1.48 (which can be downloaded from http://imagej.nih.gov/ij/download.html), band intensities in gel pictures were densitometrically calculated, and the results were then further

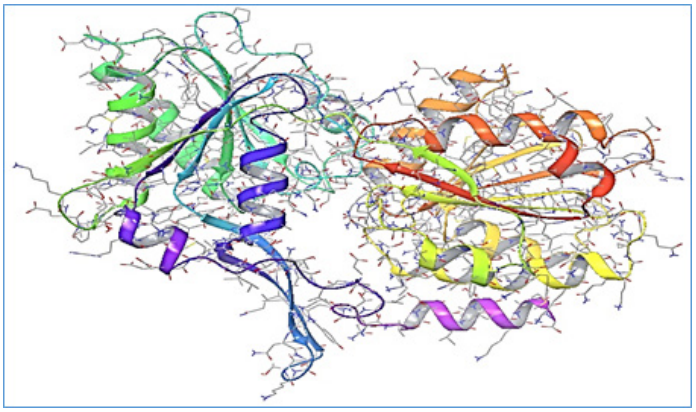

Figure 1: DHX8 Protein (Helicase ATP Binding Domain).

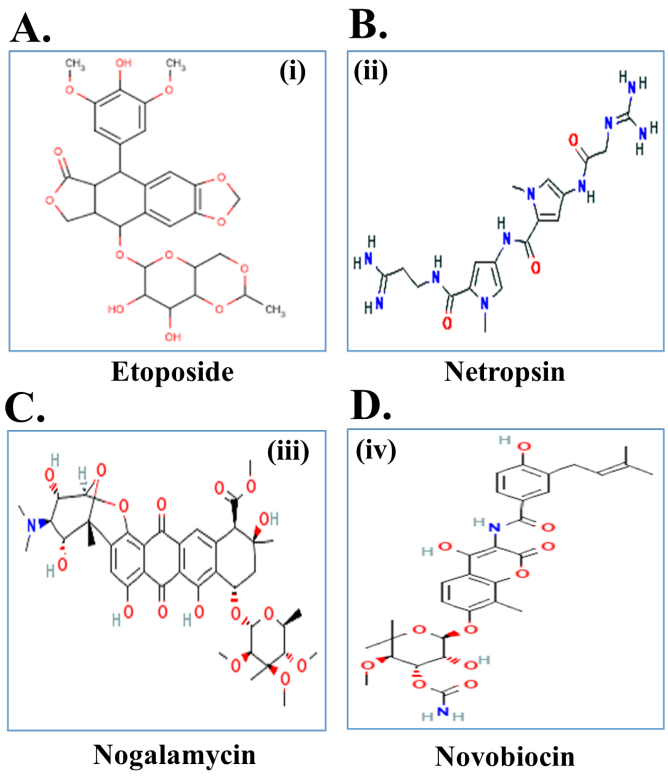

Figure 2: Structure of test compounds.

analyzed in Excel. Plotting reaction rates vs. time graphs were done using GraphPad Prism.

RESULTS

Molecular docking

Molecular docking was performed to establish the binding ability of the test compounds with the target protein DHX8 (Figure 3A and B). The docking scores of test compounds are enlisted in Table 1. From the four tested compounds, novobiocin (Pubchem Id 54675769) was found to show maximum binding capacity with target protein (DHX8_model_01.pdb) and having docking score of -9.43 Kcal/mol compared to other test compounds (Figure 3A(iv); Table 2). Docking of test compounds into the active site of target protein exhibited different molecular interactions (hydrogen bond, pi-pi interaction, and hydrophobic interaction) and might be considered responsible for the observed activity of the compounds. Novobiocin exhibited hydrogen bond interactions with target protein residues (Val 1, Tyr 27, Arg 166 and Asp 275) and other Van der Waal interactions with target protein residues (Ala 3, Val 4, Leu 10, Val 12, Ile 24, Leu 28, Ile 140, Phe 161 and Ile 163) (Figure 3A(iv)).

Fluorescence Emission Spectra (FES) of novobiocin and DHX8

To understand the change in tertiary structure by observing the intrinsic fluorescence of aromatic amino acid (tryptophan) residues present in DHX8, we next measured the fluorescence emission intensity of DHX8 alone and DHX8 bound to novobiocin at 280-500nM. The fluorescence emission intensity of DHX8 linked to novobiocin was significantly lower than that of DHX8 alone (Figure 4). These findings confirm the binding and inhibition of DHX8 by novobiocin. In addition to this, results also showed that 2.5μM concentration is adequate to inhibit the activity of DHX8.

ATPase assay

The tests on the ATPase enzyme used this DHX8 protein. Using 25, 50, and 100 nM proteins, the RNA-dependent ATPase movement's concentration dependency was examined, and the amount of released Pi (radioactive inorganic phosphate) from [32P] ATP was calculated. The experiment results show that the protein has concentration-dependent ATPase activity (Figure 5A). A 25 nM enzyme (Protein, DHX8) hydrolyzes ATP by around 15%, whereas a 50 nM enzyme releases about 25%. A 100nM concentration of the protein DHX8 resulted in maximal hydrolysis of ATP of about 30%. The outcomes show that DHX6's ATPase activity was noticed.

Effect of Novobiocin on Enzyme

The ATPase activity of DHX6 (100 nM) was assessed for additional enzymatic activity in the presence of 20 μM of DNA-binding ligands (novobiocin). The findings show that novobiocin strongly inhibits DHX6's ATPase activity in a concentration-dependent manner (Figure B). The ATPase activity inhibition research of DHX6 was conducted using this ligand at increasing concentrations (0.5 to 20.0 M), and the effective inhibitor (novobiocin) is evident at IC_{50}: 5.0987 values (Figure 5B).

Enzyme Kinetics

For kinetics analysis with Novobiocin as the ligand. A control lacking an enzyme is Lane C. Lane 1 is an uninhibited positive control, lane 2–5 with an enzyme and an inhibitor with a rising substrate concentration (0.2-2.0mM). The results unmistakably show that the *Km* values (4.930e-032) and V_{max} values (-3.042e+014 to 120194) drastically decreased in the presence

Table 1: Docking Results of Compounds with DHX8 Protein.

Compounds Ids	Compound Name	Docking score (KJ/mol)
54675769	Novobiocin	-9.4
4461	Netropsin	-7.0
5289019	Nogalamycin	-4.7
36462	Etoposide	-3.0

Table 2: Docking Results of Compounds with DHX8 Protein.

Compound Name	H-bonds forming residues	Other Interactions
Novobiocin	Val 1; Tyr 27; Arg 166 and Asp 275	Ala 3; Val 4; Leu 10; Val 12; Ile 24; Leu 28; Ile 140; and Ile 163
Netropsin	Gly 17; Thr 22 and Glu 59	Ala 48; Val 52; Phe 251 and Leu 272
Nogalamycin	Gln 23; Glu 30 and Glu 59	Tyr 27 and Phe 60
Etoposide	Lys 54; Arg 55 and Asp 275	Phe 251; Pro 253; Ala 254 and Ile 274

of Novobiocin inhibitor/ligands. These values are in hydrolyzed ATP per minute (Figure C). The *Km* and V_{max} values have dropped when inhibitors are present, indicating that the inhibition is most likely uncompetitive, according to these findings.

DISCUSSION

The activity and role of DHX8 has been determined by various studies.[11,12] These studies have determined the association of DHX8 with several important biological processes such as hematopoiesis. The impairment in the functioning of DHX8 has been reported in certain diseases including cancer.[11,12,14] it has also been documented that this protein is also required for the replication of human immunodeficiency virus type 1. Further, high levels of DEAH-Box Helicases have been reported to correlate with poor prognosis of hepatocellular carcinoma.[14] All these findings together suggest that DHX8 can be considered as therapeutic target several diseases.

The bioinformatics techniques have been the most effective methods for identifying biologically active hits against molecular targets.[22,23] Use of these techniques has widened the research area towards novel drug discovery in disease horizons. In the current study we employed molecular docking to determine the binding affinity of DHX8 with four test compounds namely etoposide, netropsin, nogalamycin, and novobiocin. Molecular Docking (MD) is a widely used structure-based bioinformatics technique for the identification of drugs against target molecules.[24-27] Molecular docking makes it possible to find novel therapeutic compounds and anticipate ligand-target interactions at the molecular level.[28-32] Among the four test compounds, novobiocin

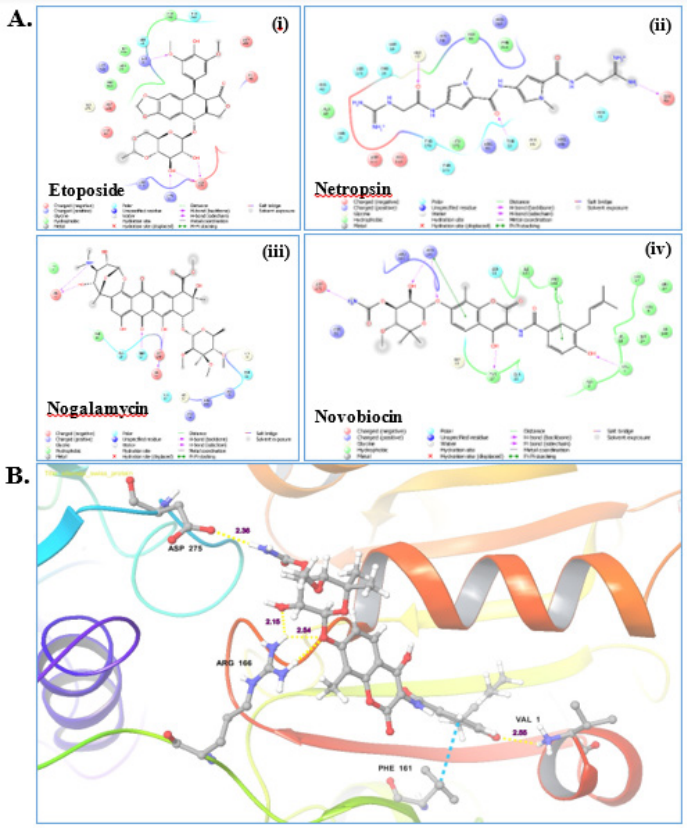

Figure 3: Docking of DHX8 with four test compounds.

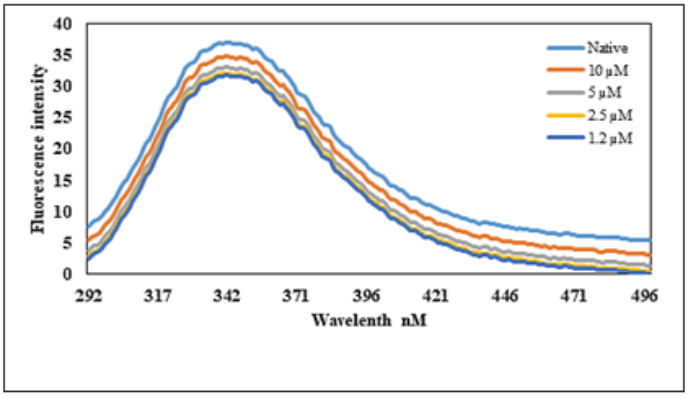

Figure 4: Fluorescence emission spectra of native protein and in presence of novobiocin.

exhibited higher binding affinity with the target protein DHX8. Novobiocin was found to be most potent compound with docking score of -9.4 Kcal/mol. Novobiocin exhibited five hydrogen bonds with four residues namely Val 1, Tyr 27, Arg 166, and Asp 275 and one Pi-Pi interaction with residue Phe 161 of the target protein DHX8. Novobiocin also assumed favorable orientation within the receptor binding site of target protein exhibiting several types of weak interactions which might be responsible for its inhibitory activity against DHX8. Further, the *in-vitro* fluorescence emission spectra of native DHX8 and DHX8 in presence of novobiocin confirmed the inhibitory effect of novobiocin on the DHX8 activity. In addition to this

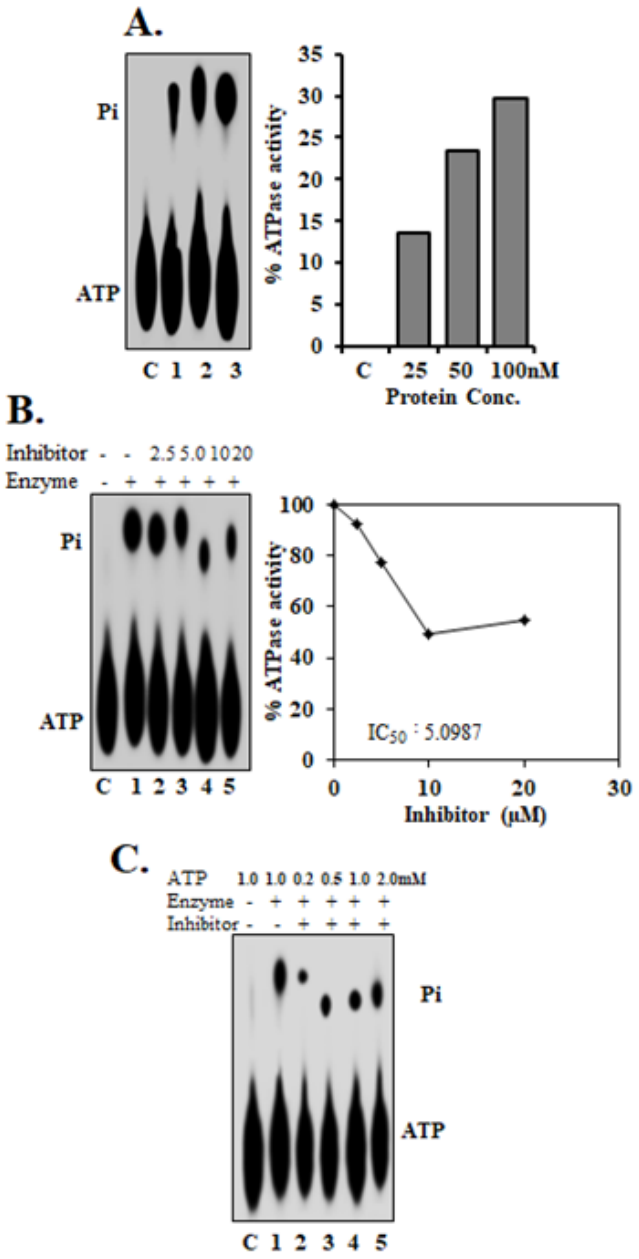

Figure 5: (A) ATPase activity of protein. The bar diagram represents the autoradiogram which shows the %ATPase activity of protein. (B) The autoradiogram for ATPase activity in the presence of ligands is shown in the left panels, and the concentration curve (Lane 2-5) is shown in the right panels. Lane C is control without enzyme (Protein) and lane 1 is reaction with enzyme without the addition of ligand. (C) Kinetics analysis in the presence of ligand Novobiocin. Lane C is control without enzyme. Lane 1 is positive control without inhibitor. Lane 2-5 with enzyme and inhibitor with increasing concentration of substrate.

we also found that 2.5µM concentration is adequate to inhibit the activity of DHX8. The results of docking and fluorescence emission spectra together revealed that DHX8 has good binding preference for novobiocin and 2.5µM concentration can be to inhibit the activity of DHX8. ATPase assay and its inhibitor study also reveal that the Novobiocin inhibits the enzymatic activity. Our study demonstrates that since both DHX6 can be targeted with the novobiocin ligand, they can be employed as suitable therapeutic targets.

CONCLUSION

DHX8 is a crucial DEAH-box RNA helicase involved in most important biological functions including homeostasis and RNA splicing. The impairment in the functioning of DHX8 has been reported in certain diseases including poor prognosis of hepatocellular carcinoma. Studies have suggested that DHX8 can be considered as target molecule in diseases including cancer. Findings of the current study provided in-depth understanding of the inhibition of DHX8 and contribute insights towards the development of novobiocin as therapeutic molecule against the DHX8 in targeted diseases. Hydrolysis assay and its inhibition result also reveal that novobiocin suitable ligand therapeutic targets. Based on the findings of current study, we conclude that novobiocin can be validated and developed as anti DHX8 molecule.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DHX8: DEAH-box helicase 8; **WH:** Winged-helix; **OB:** Oligonucleotide binding; **FES:** Fluorescence Emission Spectra; **MD:** Molecular docking.

SUMMARY

The DHX8 gene in humans encodes a protein called DEAH-box helicase 8. A member of the DEAH box polypeptide family is this protein. The conserved motif DEAH is this group's defining feature. We evaluated four different ligands with DHX8 and will summarize the report in this publication. Novobiocin has been discovered as a possible inhibitor among them. Enzymatic assay and its inhibition result also conceal that novobiocin appropriate ligand therapeutic goals. The results of this study gave a thorough understanding of how DHX8 is inhibited and contributed knowledge towards the development of novobiocin as a therapeutic molecule against DHX8 in specific disorders.

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