Objective: In recent decades, the overexpression of microRNA-21 (miR-21) is found to be progressively linked with many diseases such as different types of cancers, cardiovascular diseases, and inflammation. Thereby, it has become an attractive target for pharmacological and genetic modulation in various diseases, and also for overcoming the resistance to chemotherapy in several cancers. Here, in this study, the role of molecular therapeutics of 3,3′-diindolylmethane (DIM) has been investigated for its ability to bind with the precursor miRNA as a target of miR-21 (hsa-mir-21), which may alter the catalyzation process of dicer, a RNase III enzyme, involved in miRNA transcription. Methods: In this context, the present study describes the potential binding and the structure alteration properties of DIM to precursor miR-21 (pre-miR-21) through Molecular Docking and Molecular Dynamics simulation techniques. Results: As a corollary, DIM formed both non-bonded and covalent interactions with the bases of pre-miR, while covalent interaction with guanine in the 6th position was found to be consensus in molecular dynamics simulation. Furthermore, the stability of both DIM and pre-miR-21 was found to be inversely correlated to each other in binding condition. Conclusion: This result indicates that DIM can be used in target-based therapy and also as a lead for further development of potent small molecule miRNA antagonist.

Keywords: 3,3′-Diindolylmethane, cancer, miR-21, molecular docking, molecular dynamics simulation, pre-miR-21
concluded that miR-21 may function as an oncogene in various human cancers.\cite{7,9,11,21,29}

In traditional theory, oligonucleotide-based gene therapy approaches such as synthetic miRNA, antagonim, and miRNA sponge/decoy are usually used for the miRNA functional studies and for therapeutic intervention.\cite{26,27} Nevertheless, due to their limitations such as chemical instability, cellular delivery, and inappropriate biodistribution,\cite{28} nowadays, other techniques based on small molecular chemical biology have been followed.\cite{29-33} Particularly, small molecules may directly bind to the miRNA and alter its functions. In general, most of the miRNA are formed in cell by the dicer-catalyzed processing of their corresponding inactive precursor miRNA (pre-miRNA) and this step has been proposed as an appropriate target for inhibitor design.\cite{34-38} Furthermore, high affinity synthetic small molecule ligands have been recently described to bind with pre-miRNA.\cite{38-40} In this regard, we aim to analyze the binding properties of 3,3′-diindolylmethane (DIM) with pre-miR-21, as a potential inhibitor of miR-21. DIM is a natural compound that is abundant in cruciferous vegetables such as broccoli, cauliflower, and cabbage, and it is a major acid condensation product of indole-3-carbinol.\cite{41} DIM has been demonstrated to reduce the expression of certain miRNAs. It inhibits cancer cell growth by controlling cell proliferation, transcription, and apoptosis through regulation of gene expression. Moreover, it is evident that it reverses the expression level of oncogenic miRNAs by inhibiting the expression of miR-21.\cite{42} Results showed that the inhibition of endogenous miR-21 is specific, efficient in the treatment of cancer.\cite{42}

Coupled with that, the present study is designed with some computer-aided drug discovery methods such as molecular docking and molecular dynamics simulation to investigate the bindings and the changes in the dynamics behavior of functional region of pre-miRNAs. As we know that conformational changes in the protein or macromolecules structure affect its biological function, and also it is evident that the conformational flexibility of a macromolecule influences the interactions with ligand or its biological partners at different level.\cite{42,43}

**Materials and Methods**

**Data and databases**

The data from databases used in this study include protein data bank (PDB)\cite{44} and PubChem.\cite{45} PubChem is a public repository of small molecules and their biological properties. Currently, it contains more than 25 million unique chemical structures and 90 million bioactivity outcomes associated with several thousand macromolecular targets.\cite{46}

**Ligand selection and preparation**

For docking studies, compound was retrieved from Pubchem databases, i.e., DIM (CID 3071). The 3D structure for this compound was built by using Ligprep2.5 in Schrödinger Suite 2013 with OPLS_2005 force field. Their ionization states were generated at pH7.0 ± 2.0 using Epik2.2 in Schrödinger Suite. Up to 32 possible stereoisomers per ligand were retained.

**MicroRNA structure preparation**

The NMR solution structure of pre-miR-21 having lowest energy was obtained from (pdb id: 2MNC).\cite{44} The solution structure of pre-miR-21 (CCGUUGAAUCUCACGG) was prepared and refined by using the Protein Preparation Wizard of Schrödinger-Maestro v9.4. Charges and bond orders were assigned, hydrogens were added to the heavy atoms, and all waters were deleted. It was optimized at neutral pH. Using force field OPLS_2005, minimization was carried out setting maximum heavy atom root mean square deviation (RMSD) to 0.30 Å.

**Docking simulation study**

Before docking, the structures were prepared by using dock preparation wizard integrated in UCSF Chimera,\cite{47} by adding hydrogen and Gasteiger charges.\cite{48} Input ligand file format was MOL2, so these were further converted into pdbqt format by Python Molecular Viewer with AutoDock tools, required by AutoDock Vina.\cite{49} The size of grid box in AutoDock Vina was kept at 30.72, 25.00, and 25.00, respectively, for X, Y, and Z. The energy range was kept at 4 which was default setting. AutoDock Vina was implemented through shell script provided by AutoDock Vina developers. The binding affinity of ligand was observed by Kcal/Mol as a unit for a negative score.\cite{50}

**Molecular dynamics simulation**

Molecular dynamics simulation was performed using the NAMD\cite{51} engine integrated in MOE software. In this study, the AMBER12:EHT (Extended Huckel Theory Group II ion and Group VIII parameters OPLS-AA) force field was utilized, as it is widely applied to describe macromolecular system. The system was solvated and neutralized by adding Cl⁻ and/or Na⁺ ions and water molecules, where the total solvent molecules was 1827; 1.023 g/cm³. The periodic boundary condition was employed to perform the simulation, where the box size was 43.3 Å × 38.0 Å × 38.1 Å. Following steepest descent energy minimization, equilibration of 100 steps done in NPT ensemble with Periodic Boundary.
Conditions, Particle Mesh Ewald for full-system periodic electrostatics, and constant temperature dynamics through Langevin Dynamics, constant pressure dynamics through Nose-Hoover Langevin piston, and SHAKE was used to maintain all bonds involving hydrogen atoms at their equilibrium values. Finally, the full system was subjected to molecular dynamics (MD) production run at 300 K temperature for 10 ns in NVT ensemble.

Results and Discussion

Docking simulation study

Molecular docking is one of the most computational techniques, which not only predicts the binding affinity of a ligand to a receptor but also demonstrates the binding mode in the particular active site. We therefore performed docking analysis to understand the molecular interactions of DIM in binding with miR-21. In essence, DIM formed three conventional hydrogen bonds with \( U_{11}, \ U_{12} \) bases, where a hydrogen bond was formed with the oxygen atoms of 11th uridine and rest of two were formed with another oxygen atom of 12th uridine. Moreover, a \( \pi-\pi \) interaction was observed between the indole ring and the purine ring of 20th adenine. A detailed description of the DIM binding with miR-21 is demonstrated in Table 1 and also rendered in Figure 1. According to the docking analysis, the binding affinity of DIM was found to have -7.3 kcal/mol, which may indicate a strong binding with pre-miR-21. On this side, analysis of complex stability was carried out using long MD simulations to support the docking prediction, which is described in further sections.

| DIM-pre-micro RNA-21 | Hydrophobic | Hydrogen bond |
|----------------------|-------------|---------------|
| Docked               | A\( ^{b} \) (5.9604) | - |
| Confirmation         | U\( ^{11} \) (2.19432) | U\( ^{12} \) (2.80226) |
| 1 ns                 | U\( ^{4} \) (2.84371) | U\( ^{4} \) (1.82696) |
|                      | A\( ^{13} \) (2.58905) | G\( ^{4} \) (2.02535) |
|                      | A\( ^{13} \) (2.62459) | A\( ^{13} \) (1.69988) |
| 3 ns                 | U\( ^{4} \) (2.83271) | A\( ^{13} \) (2.29783) |
|                      | A\( ^{13} \) (2.29783) | |
| 5 ns                 | U\( ^{4} \) (2.89161) | U\( ^{4} \) (2.21209) |
|                      | C\( ^{14} \) (2.5152) | G\( ^{4} \) (2.29273) |
|                      | U\( ^{4} \) (2.97087) | |
|                      | C\( ^{14} \) (2.95862) | |
| 8 ns                 | G\( ^{6} \) (2.53119) | G\( ^{6} \) (2.09141) |
|                      | A\( ^{13} \) (2.74835) | |
| 9 ns                 | G\( ^{6} \) (2.85894) | G\( ^{6} \) (2.1496) |
| 10 ns                | G\( ^{4} \) (2.52847) | - |

DIM: 3,3′-Diindolylmethane

Figure 1: Interactions and binding pose of 3,3′-diindolylmethane with precursor microRNA-21 complex were generated from AutoDock Vina. Here, green dots represent hydrogen bonding, while pink represent \( \pi-\pi \) T-shaped.
**Molecular dynamics simulation**

To impart stability, the pre-miR-21-DIM complex was subjected to a MD simulation for 10 ns. The energy profile of the system such as the potential energy and kinetic energy and the other factors such as temperature and pressure are rendered in Figure 2. As described in Figure 2, it can be concluded that the system had been reached at equilibration and remained stable in 10 ns simulation. Moreover, the volume of the system remained constant at the time of simulation, which is 62610.11 Å$^3$. To describe the stability and flexibility of the ligand binding, we calculated the RMSD profile of both miR-21 and DIM.

**Figure 2:** Stability of the simulation system. Stability was evaluated in terms of the (a) potential energies, (b) kinetic energies, (c) pressure, (d) temperature of the simulation as a function of simulation time.
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Along with the time steps. As can be seen in Figure 3, the RMSD of the backbone of miR-21 increased from ~1 Å at 0 ns to 3 Å at 1 ns and remained stable at 2–3 Å for 3.75 ns. After that, it then increased to 4 Å and remained stable for 8 ns. However, following 8 ns, the RMSD is tending to decrease, which means that the miRNA is somewhat stable in the simulation, afterward. In contrast, the RMSD of DIM remained stable at ~1 Å, from 0 ns to

Figure 3: Backbone root mean square deviations are shown as a function of time for precursor microRNA-21 and 3,3′-diindolylmethane at 300 K. Here, precursor microRNA-21 is shown in black and 3,3′-diindolylmethane in red

Figure 4: Two-dimensional representation of molecular interactions between the precursor microRNA-21 and 3,3′-diindolylmethane, complexes were sampled from molecular dynamics simulation. The interactions were rendered by Ligplot
8.5 ns. Nevertheless, it increased to ~2 Å and seems to lose its stability afterward. Interestingly, it can be assumed from the RMSD plot that in binding with DIM, the pre-miR-21 was less stable for 8 ns, while DIM was found to be more stable at that time. In this manner, after 8 ns, the stability of DIM was decreased, while the stability of pre-miR-21 increased. To justify this hypothesis, we examined the molecular interactions of the complex in different snapshot of MD simulation. According to Table 1 and Figure 4, it is observed that the DIM formed conventional hydrogen bonds with UÅ and GÅ bases in 1 ns and 5 ns snapshot and also had covalent interactions by forming carbon–hydrogen bonds with UÅ, AÅ, and CÅ bases. Similarly, in 8 ns and 9 ns simulation snapshot, GÅ base was involved in both noncovalent and covalent interactions with DIM; however, only one covalent interaction remained in 10 ns simulation. As a corollary, the number of bonding interaction was gradually decreasing by the increasing of time step in the simulation, which means that increasing of DIM flexibility after 8 ns is due to the breaking down of its bonding interactions with pre-miR-21.

Our findings from the molecular docking and molecular dynamics simulation studies suggested that DIM has the potential chemical affinity and ability to alter the stability of pre-miRNA of miR-21. These results postulate DIM as an important ligand of potential importance for the development of potent small molecular miRNA antagonist and the remedial approaches in various cancers.

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Conflicts of interest
There are no conflicts of interest.

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