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In silico and *in vitro* validation of some benzimidazole derivatives as adenosine deaminase inhibitors

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Benzimidazole nucleus considered as an important scaffold for developing drug candidates against a wide spectrum of diseases. Adenosine deaminase (ADA), an enzyme present in purine metabolic pathway, has a significant role in inflammatory and malignant diseases and hence it is considered as a major target for drug development. The present study investigates ADA inhibitory potential of selected benzimidazole derivatives by using *in silico* and *in vitro* methods. Molecular docking and dynamics simulations have been carried out to identify potential ADA activesite binders from benzimidazole derivatives. Compounds having strong binding affinities were selected for enzyme inhibition assays and fluorescent binding studies. The results showed that the pyridinyl and butyl derivatives of benzimidazole possess significant ADA inhibitory potential.The study proposes these compounds can be used as potent candidates for developing ADA inhibitor drugs.

Keywords: Adenosine deaminase, benzimidazole, inhibition assay, fluorescence spectroscopy, molecular dynamics

Benzimidazoles are hetercyclic organic compounds containing imidazole moiety fused to benzene ring. Benzimidazole is an important pharmacophore and a privileged scaffold in medicinal chemistry[1]. It has been observed that, drugs belonging to different groups such as antimicrobials, antioxidants, anti-inflammatory, immunomodulators, and antidiabetics contain benzimidazole moiety. Thiabendazole, flubendazole (anthelmintic), astmizole (antihistaminic), lansoprazole and omeprazole (antiulcerative) are some examples of marketedbenzimidazole drugs.

Adenosine deaminase (ADA), also known as adenosine aminohydrolase, is a key enzyme involved in the conversion of adenosine to inosine irreversibly[2]. ADA is a zinc containing metalloenzyme present in both prokaryotes and eukaryotes. In humans, ADA is normally present in all tissues, highest activity observed in lymphoid system[3, 4]. ADA plays a crucial role in adenosine homeostasis and nucleic acid metabolism. The binding of ADA with CD26 and adenosine receptors triggers immune responses and various cellular activities[5-7]. Deficiency of the enzyme leads to severe combined immune deficiency (SCID)[8]. Over-activity of ADA leads to inflammation and certain types of cancers[9, 10]. The interaction of extracellular form of ADA (ecto-ADA) with CD26[11] regulates T cell activation and chronic inflammation by metabolizing extracellular adenosine released at inflamed sites, which are toxic to lymphocytes[12]. The role in regulating immune responses and cell growth make ADA a potential drug target against various diseases. Moreover ADA inhibitors getting increased attention as immuno-suppressants[13]. Due to the involvement of ADA in various pathogenesis, its inhibitors are being considered as promising candidates for the development of anti-inflammatory, anti-proliferative and immune-suppressant drugs[14].

Till date numerous ADA inhibitors have been reported from natural and synthetic sources. Most of them have several drawbacks including toxicity, low potency and poor pharmacokinetics. The main objective of the current study was to investigate ADA inhibitory potential of some benzimidazole derivatives by using *in silico* and *in vitro* methods.

Materials and Methods

Docking Studies

The ADA active site binding property of benzimidazole derivatives was studied by *in silico* methods. Schrodinger suite (Maestro) was used for the molecular docking studies[15]. The crystal structure of bovine ADA in complex with EHNA (pdb ID:2Z7G) from PDB was used as the protein input structure. The structure was prepared for docking

studies using the protein preparation wizard of Schrodinger software using OPLS_3 force field[16]. A grid of dimension 12 Å was generated around the bound ligand, EHNA in the crystal structure in such a manner that it could cover the entire active site cleft. The structure data files (SDF) of benzimidazole derivatives were downloaded from ZINC database (http://zinc.docking.org/). The downloaded ligand files were prepared using ligprep module of Schrodinger suite. Docking simulations were carried out using extra precision (XP) method of glide module.

Molecular Dynamics

MD simulations were conducted using Desmond module of Schrödinger (Schrödinger, LLC, New York) to assess the binding stability of benzimidazole derivatives at the active site of ADA[17]. The high scored ligand docked ADA complexes, were used as the initial structure for MD studies. An orthorhombic water box was generated in such a way that it covers the entire protein-ligand complex using TIP3P solvent model. Based on the charges of each complex, sodium or chloride ions were added accordingly to neutralize the charge of complexes. The solvated systems for molecular dynamics were generated using system builder option in Desmond module. After system generation, minimization and relaxation of the protein-ligand complex under NPT ensemble,was carried out using default protocol of Desmond module. This process includes a total of 9 stages among which there are 2 minimization and 4 short simulations (equilibration phase) steps before starting the actual production time.

The stages that includes in MD run;

Stage 1 – task

stage 2 - minimize, Minimization with restraints on solute

stage 3 - minimize, Minimization without any restraints

stage 4 - simulate, Berendsen NVT, T = 10 K, small timesteps, and restraints on solute.heavy atoms

stage 5 - simulate, Berendsen NPT, T = 10 K, and restraints on solute heavy atoms

stage 6 - solvate_pocket

stage 7 - simulate, Berendsen NPT and restraints on solute heavy atoms

stage 8 - simulate, Berendsen NPT and no restraints

stage 9 – simulate

Molecular dynamic simulations were conducted with the periodic boundary conditions in the NPT ensemble using OPLS_3 force field parameters. The temperature and pressure were kept at 300 K and 1 atmospheric pressure respectively using Nose-Hoover temperature coupling and isotropic scaling[18]. The operation was followed by running the 30 ns NPT production simulation.

Enzyme inhibition assay

ADA (bovine), Benzimidazole derivatives and adenosine were purchased from Sigma Aldrich. The ADA inhibitory activity of selected benzimidazoles was determined through Berthelot reaction[19]. In this reaction, ADA converts adenosine to inosine and produce ammonia. This ammonia reacts with phenol and sodium hypochlorite to form indophenol. The quantity of indophenol was determined spectrophotometrically by measuring the optical density (OD) at 635 nm. The reaction was initiated by the addition of 10 µL enzyme solution (0. 40 units/ml) to the reaction mixture prepared in the 50 mM potassium phosphate buffer of pH 7.4 containing 20 µM adenosine. After five minutes, the reaction was stopped by the addition of 400 µL phenol-nitroprusside solution. To this solution 500 µL of sodium hypochlorite in 0.6 M NaOH was added and incubated for 30 minutes. The OD at 635 nm was measured using a UV spectrophotometer (Thermo Fisher Scientific, MA, USA). A solution without the enzyme was taken as the blank for the measurement of OD. EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine), a potent inhibitor of ADA was used as positive control. The ADA activity was expressed in micromoles of ammonia liberated in one minute. The assay was repeated in triplicate under same conditions. The enzyme kinetics studies were carried out with different substrate concentrations (10-60 µM). The Lineweaver-Burk plot was drawn and Michaelis-Menten constant (Km) and Maximal Velocity (Vmax) were determined from the graph.

Fluorescence spectroscopy

The interaction of benzimidazole derivatives with ADA active site was studied using Fluorescence spectroscopy with varying ligand concentration. Fluorescence measurements were carried out using HORIBA FluoromaxSpectrofluorometer (Kyoto, Japan) with a fluorescence cuvette of 1.0 cm path length. The fluorescence emission of bare ADA and

that with different concentrations of the ligands were recorded at room temperature. Excitation wavelength was set as 290 nm and emission was scanned every 1 nm in the range of 300 nm to 400 nm

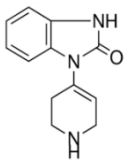
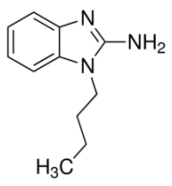
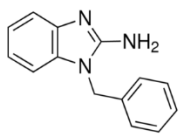
Results and Discussion

Computational screening of ADA inhibitors

The *in silico* docking studies revealed that some compounds among the screened benzimidazolederivatives have the potential to be ADA inhibitors. Three such compounds, 1,3-Dihydro-1-(1,2,3,6-tetrahydro-4-pyridinyl)-2H-benzimidazole-2-one (Zinc ID: 394330), 1-Butyl-1H-benzoimidazol-2-ylamine (Zinc ID: 2475956) and 1-Benzyl-1H-benzoimidazol-2-ylamine (Zinc ID : 132982) were selected for further studies. The molecular structures and binding scores are shown in Table I.

Zn 394330, benzimidazole derivative with pyridinyl ring, showed the highest binding affinity towards ADA active site. The compound formed hydrogen bond with the enzyme residue Asp 16 and pi-pi staking interaction with Phe 58. The glide score obtained was -7.267 Kcal/mol. Another selected benzimidazole derivative having a butyl chain, Zn 2475956, showed a glide score -6.43 Kcal/mol. This compound formed hydrogen bonds with the residues Asp 16 and Asp 293. The third selected derivative, Zn 132982 madehydrogen bonds with residues Gly 181 and Glu 214. The glide score for binding was -4.786 Kcal/mol.

Table I — The molecular structures and binding scores of the selected benzimidazole derivatives.

Compounds	Molecular structure	Glide score (Kcal/mol)	Enzyme residues that make hydrogen bonds with the ligands
Zn 394330		-7.267	Asp 16
Zn 2475956		-6.43	Asp 16, Asp 293
Zn 132982		-4.786	Gly 181, Glu 214

Molecular Dynamics

The binding stability of top scored benzimidazole derivatives at ADA active site was assessed through molecular dynamics studies by using Desmond module of Schrodinger software. The deviation from initial structure was expressed in terms of Root Mean Square Deviation (RMSD). The results showed that the binding of the compounds with ADA were stable and no drastic change from the initial position was observed during the 30 ns MD run (Figure 1). The RMSD observed was below 3 Å. The ADA-Zn

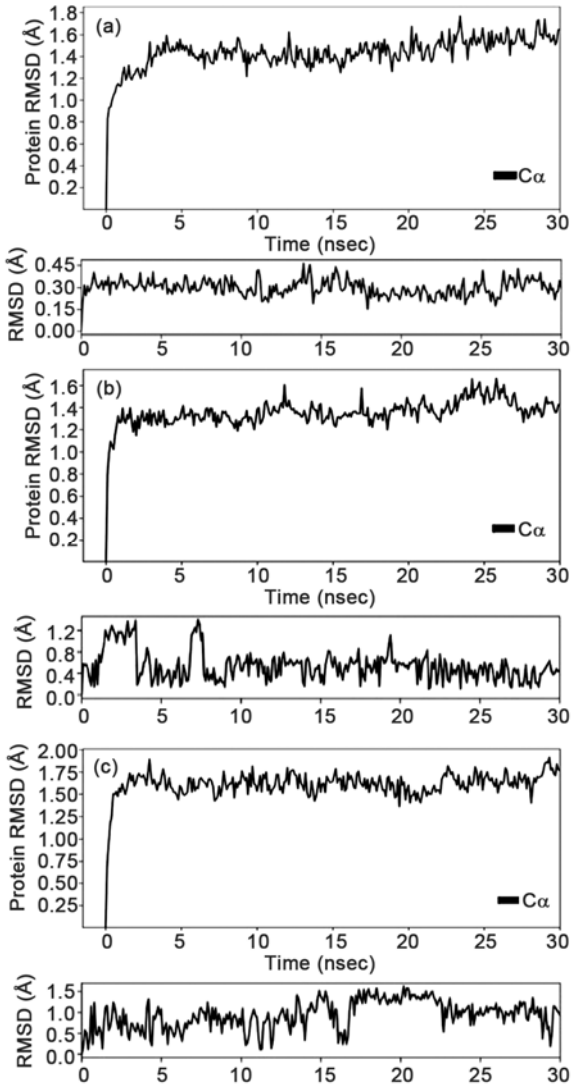


Figure 1 — Protein (top) and ligand (bottom) RMSD change observed for each complex, Zn 394330 (a), Zn 2475956(b) and Zn 132982 (c) during 30 ns MD run.

394330 complex was very stable during the simulation with an RMSD below 1.8 Å for the protein and 0.5 Å for the ligand (Figure 1a). The intermolecular hydrogen bond with Asp 16 existed throughout the simulation time (Figure 2).

The complex with Zn 2475956, showed protein RMSD around 1.6 Åand ligand RMSD around 1.2 Å (Figure 1b). The hydrogen bonds that stabilized the binding, Asp 16 and Asp 293 were found to be stable during the MD run (Figure 3). The RMSDs for protein and ligand were 2 Å and 1.5Årespectively in the case of ADA-Zn 132982 complex (Figure 1c). The binding of Zn 132982 at ADA active site was mainly stabilized by hydrophobic interactions involving the phenyl ring. The majority of these hydrophobic interactions existed during MD run (Figure 4).

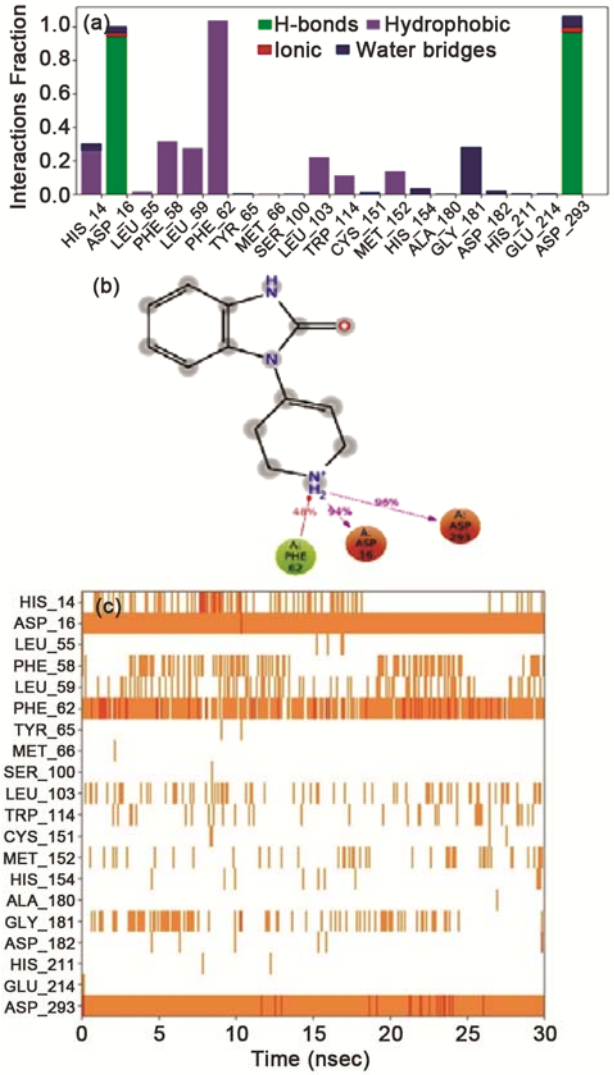


Figure 2 — Interaction details of Zn 394330 with ADA active site during the course MD simulation. (a) Protein interactions with the ligand (Zn 394330) monitored during the MD run. The y axis value 1 indicates that 100 % of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts with the ligand. (b) Schematic diagram of detailed ligand atom interactions with the protein residues and percentage of time each contact retained during the simulation time. (c) A timeline representation of the interactions and contacts observed with 394330 and ADA in each trajectory frame during the MD run.

ADA inhibition assay

ADA inhibitory activity of the selected benzimidazole derivatives was analyzed by Berthelot

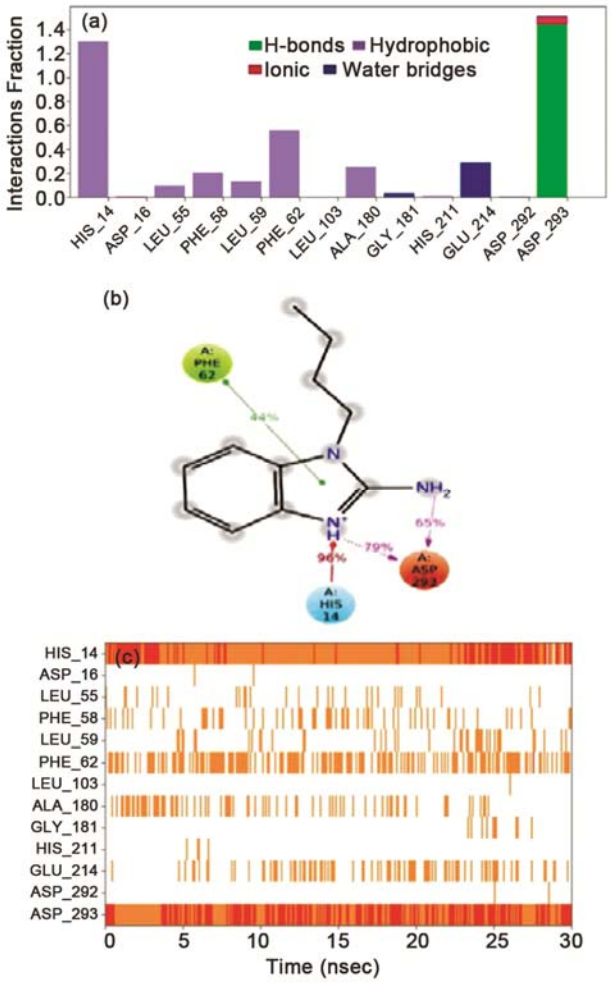


Figure 3 — Interaction details of Zn 2475956 with ADA active site during the course MD simulation. (a) Protein interactions with the ligand (Zn 2475956) monitored during the MD run. The y axis value 1 indicates that 100 % of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts with the ligand. (b) Schematic diagram of detailed ligand atom interactions with the protein residues and percentage of time each contact retained during the simulation time. (c) A timeline representation of the interactions and contacts observed with Zn 2475956 and ADA in each trajectory frame during the MD run.

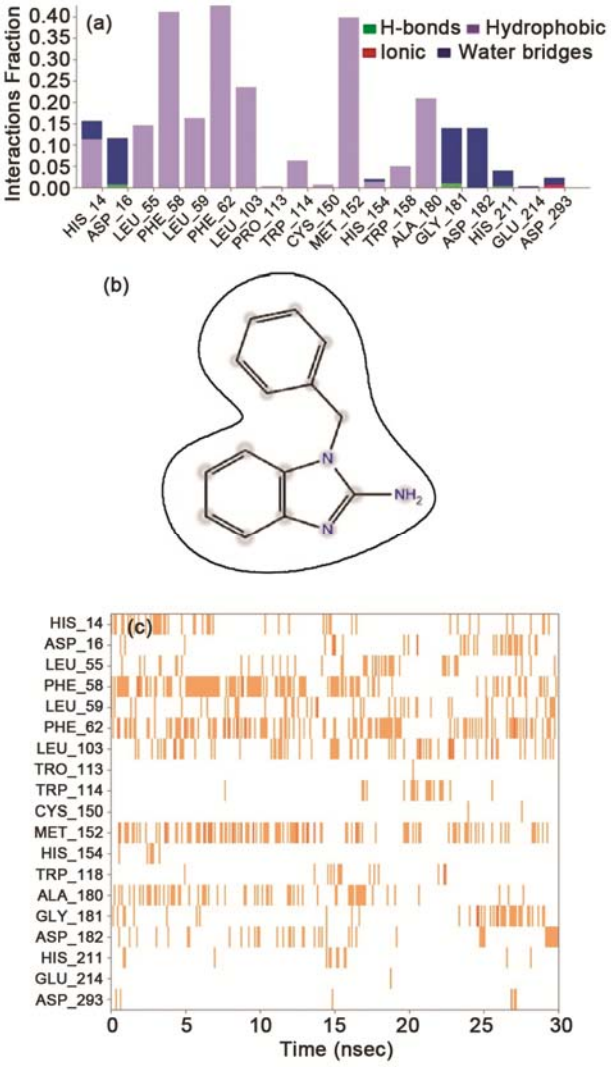


Figure 4 — Interaction details of Zn 132982 with ADA active site during the course MD simulation. (a) Protein interactions with the ligand (Zn 132982) monitored during the MD run. The y axis value 1 indicates that 100 % of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts with the ligand. (b) Schematic diagram of detailed ligand atom interactions with the protein residues and percentage of time each contact retained during the simulation time. (c) A timeline representation of the interactions and contacts observed with Zn 132982 and ADA in each trajectory frame during the MD run.

reaction. EHNA, the standard ADA inhibitor was used as the positive control. The compound Zn 394330 showed highest inhibition on ADA activity (Figure 5). The percentage of inhibition was found to be 72. Zn 2475956 showed moderate inhibition (39 %) and Benzyl-1H-benzoimidazol-2-ylamine (Zn 132982) showed weak inhibition (around 10 %) (Figure 6). The assay results showed that the

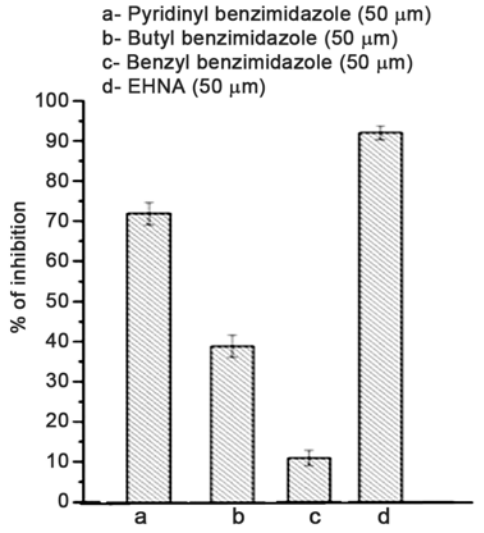


Figure 5 — ADA inhibitory activity of Zn 394330 (a), Zn 2475956 (b), Zn 132982 (c) and standard inhibitor EHNA (d).

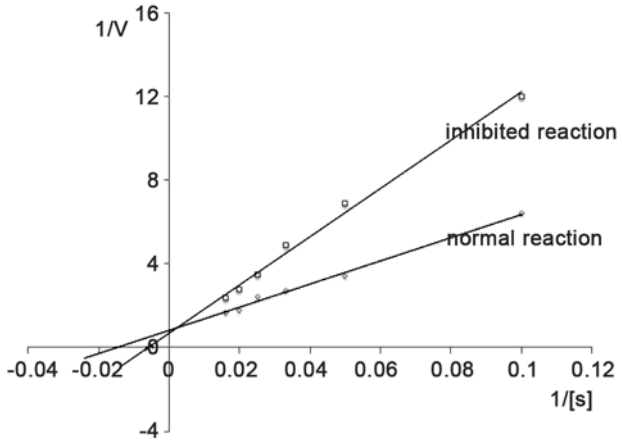


Figure 6 — Lineweaver–Burk plot of normal reaction and Zn 394330 inhibited reaction

benzimidazole with pyridinylring (Zn 394330) is a strong inhibitor of ADA while the butyl derivative (Zn 2475956) is only a moderate inhibitor. The benzyl derivative (Zn 132982) did not show any significant inhibition on ADA activity (Table II).

The kinetics of inhibition was studied using different concentration of substrates (adenosine) ranging from 10 to 60 μM. From the kinetics assay results, Lineweaver–Burk graph was plotted and Km, K'm (altered km) and Ki (inhibitor constant) values were determined. The compound Zn 394330 exhibited competitive mode of inhibition. The Ki value was determined as 20.83 μM (Figure 6). The compound Zn 2475956 also showed competitive type of inhibition and Ki obtained was 149.96 μM (Figure 7).

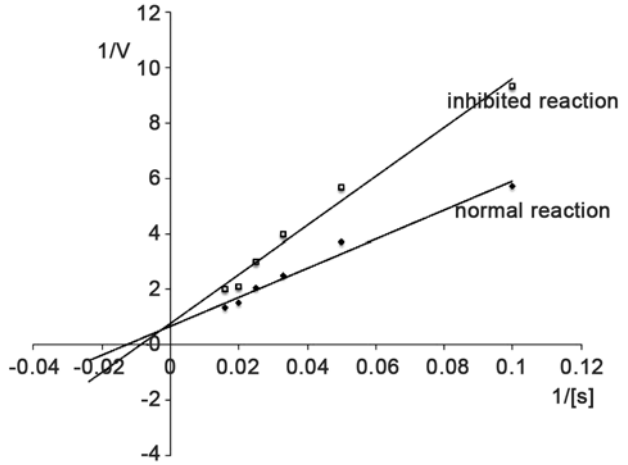


Figure 7 — Lineweaver–Burk plot of normal reaction and Zn 394330 inhibited reaction.

Table II — Inhibitor Constant (Ki) calculated from enzyme kinetics study.

Compound	Inhibitor Constant (Ki) μM
Pyridinylbenzimidazole (Zn 394330)	20.83
Butyl benzimidazole (Zn 2475956)	149.96

Fluorescence spectroscopy

The interaction of inhibitors with ADA active site was studied using tryptophan fluoresce quenching studies. The active site of ADA contains two tryptophan residues, Trp 114 & Trp 158[20, 21] and the interaction of these tryptophan residues with inhibitor on binding will lead to the quenching (reduction) of fluorescence intensity. The study was conducted with bare ADA and ADA with different concentration of ligands. The protein was excited at 290 nm and emission was recorded. As the concentration of ligand increases (2-20μM) the emission spectrum showed a remarkable decrease inthe fluorescence emission (Figure 8). This concentration dependent decrease in the fluorescence indicated that the study results showed that pyridine derivative of Benzimidazole (Zn 394330) is a very strong binder of active site.

The butyl derivative (Zn 394330) also showed significant quenching of ADA fluorescence, but not as strong as that of the pyridine derivative (Figure 9). The benzylbenzimidazole (Zn 132982) didn't show any significant quenching and hence a weak binder to ADA active site (Figure 10).

From the fluorescence emission spectrum data the Stern-Volmer plots were generated for pyridinyl and butyl derivatives using F0/F values and ligand concentration. F0 and F correspond to the fluorescence intensities of the protein without

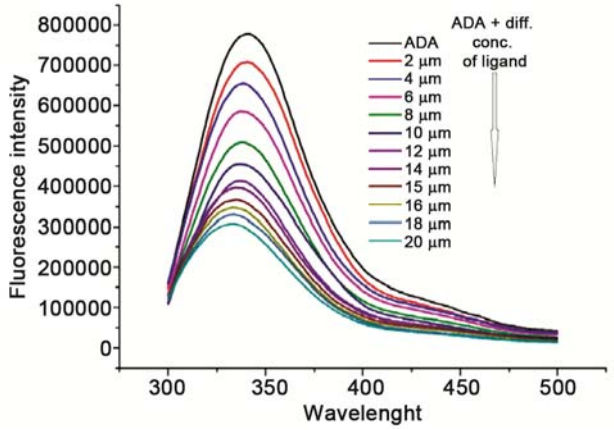


Figure 8 — Fluorescence emission spectra of ADA with increasing concentration of Zn 394330 (0–20 μM) on excitation at 280 nm.

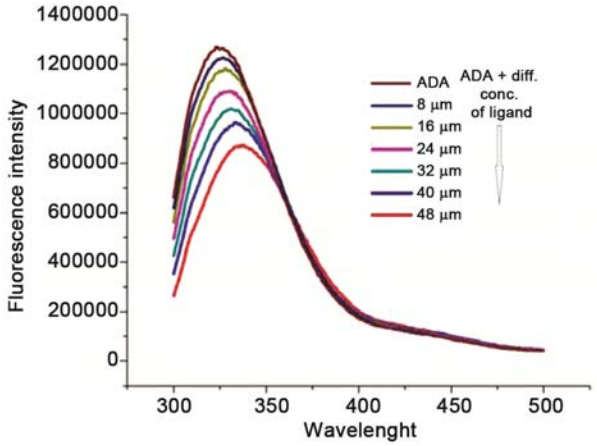


Figure 9 — Fluorescence emission spectra of ADA with increasing concentration of Zn 2475956 (8- 48 μM) on excitation at 280 nm

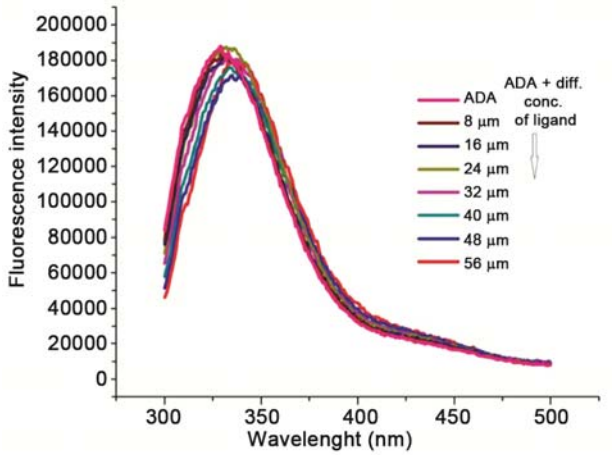


Figure 10 — Fluorescence emission spectra of ADA with increasing concentration of Zn 132982 (8- 56 μM) on excitation at 280 nm

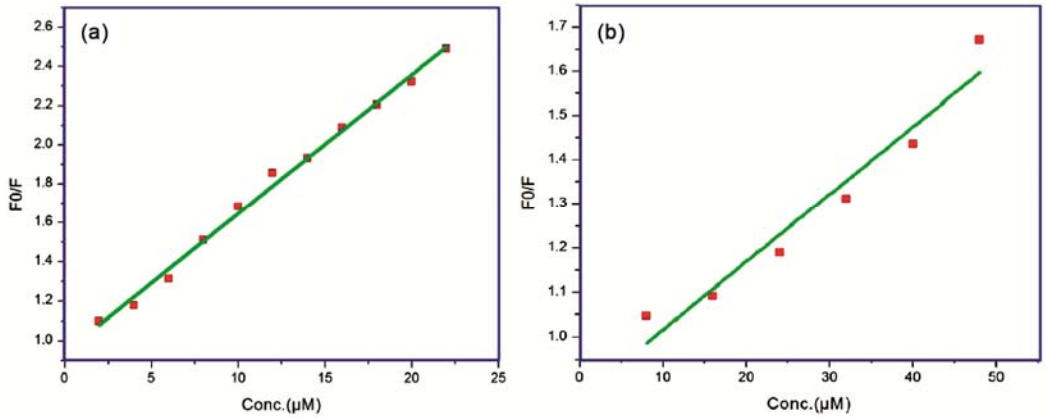


Figure 11 — Stern–Volmer plot of the ADA fluorescence quenching by Zn 394330 (a) and Zn 2475956 (b).

Table III — Stern −Volmer Constants (K_{SV}) values obtained from fluorescence quenching studies.

Compound	Stern−Volmer Constants (K_{SV}) M^{-1}
Pyrydynyl derivative (Zn 394330)	14.02×10^{6}
Butyl derivative (Zn 394330)	0.015×10^{6}

quencher (ligand) and with quencher respectively. Stern−Volmer Constants (Ksv), which represent the binding affinity between the quencher (ligand) and fluorophore (protein), were determined the slopes of the plots (Figure 11). Pyrdynylbenzimidazole derivative (Zn 394330) showed high Ksv value (14.02×10^{6} M^{-1}) than the butyl benzimidazole derivative (0.015×10^{6} M^{-1}) (Table III). In agreement with the enzyme inhibition studies, fluorescent quenching studies showed that Pyrdynylbenzimidazole (Zn 394330) with high binding affinity, butyl benzimidazole (Zn 2475956) has moderate affinity and benzyl benzimidazole (Zn 132982) with negligible binding affinity to ADA.

Conclusion

Benzimidazoles have wide applications in medicinal chemistry and it is considered as an important scaffold for developing therapeutic agents against many diseases. ADA inhibitory activity of some benzimidazole derivatives have been studied using *in silico* and *in vitro* methods. Three compounds, 1,3-Dihydro-1-(1,2,3,6-tetrahydro-4-pyridinyl)-2H-benzimidazole-2-one (Zinc ID: 394330), 1-Butyl-1H-benzoimidazol-2-ylamine (Zinc ID: 2475956) and 1-Benzyl-1H-benzoimidazol-2-ylamine (Zinc ID : 132982) were selected based on *in silico*studies and used for further in vitro studies. The inhibition assay showed that pyridinyl derivative is a strong inhibitor of ADA activity (Ki 20 μM), butyl derivative is a moderate inhibitor (Ki 149 μM) and benzyl derivative showed no inhibition. The binding studies using spectrofluorometry confirmed the active site binding potential of the first two compounds. The current study proposes 1,3-Dihydro-1-(1,2,3,6-tetrahydro-4-pyridinyl)-2H-benzimidazole-2-one as a potent lead compound for developing ADA inhibitor drugs.

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