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Synthesis and *in vitro* Evaluation of Dihydrobenzimidazole Thiopyranooxazinone Derivatives as a Potent Biological Agents

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ABSTRACT

In the present study, dihydrobenzimidazole thiopyranooxazinone derivatives were efficiently synthesized, which were further characterized and authenticated by means of TLC and different spectral analysis such as IR and ^{1}H NMR. The synthesized compounds **DPK2d2** to **DPK2d8** were screened for their *in vitro* antimicrobial, antitubercular and anticancer activities. The results showed that the titled compounds **DPK3d1**, **DPK3d2** and **DPK3d4** exhibited potent antimicrobial activity, shows a broad-spectrum activity against *Bacillus subtilis*, *Escherichia coli* (antibacterial) and *Aspergillus niger* (antifungal) as compared to ciprofloxacin and fluconazole, respectively. Compounds **DPK3d1**, **DPK3d3** and **DPK3d5** exhibited potent antitubercular activities against *Mycobacterium tuberculosis* as compared to pyrazinamide, ciprofloxacin and streptomycin. Compounds **DPK3d3**, **DPK3d4** and **DPK3d5** showed highly potent cytotoxic activity against human lung cancer cell line (A549) as compared to adriamycin. *In silico* molecular docking studies shown that all the ligands highest binding affinity range -6.7 to -8.7 for selected 1CB4 PDB of superoxide dismutase, which recognized that ligands having antioxidant activity.

KEYWORDS

Dihydrobenzimidazole, Thiopyranooxazinone, Antimicrobial activity, Antitubercular activity, Anticancer activity, Molecular docking.

INTRODUCTION

In the 20th century chemotherapy has revolutionized the treatment of infective diseases since the innovation of antibacterial dyes by Paul Ehrlich, covered the way to a great victory for human health and long life. The foremost limitation of the current treatment of communicable diseases is challenging due to resistance to antimicrobial agents and their side effects. Inadequate numbers of antimicrobials are available to treat infections caused by bacteria and fungi. Numbers of new communicable diseases have been discovered. So, there is an emergency need to develop novel drugs molecule, with fewer side effects, extended-spectrum activity improved stability for the treatment of communicable diseases. Nowadays researchers established an exciting searching new lead molecule battle against microbial infection. Patient morbidity, costs of treatment, rates of hospitalization and use of broad-spectrum agents are remarkably increased by antimicrobial resistance [1-3].

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Tuberculosis is a deadly disease usually caused by *Mycobacterium tuberculosis*. Besides, HIV infection causes a worldwide increase in TB cases. The synergistic interaction between TB and HIV causes *Mycobacterium tuberculosis* to develop resistance to multiple drugs. In addition to active TB, treatment of latent TB is also important to controlling TB because the reactivation of TB from untreated latent TB is a major source of new active TB infections. Therefore, it is essential to develop rational chemotherapeutic agents to delay the emergence of resistance and, ideally, shorten the duration of therapy of this infection [4-6].

Nowadays in the world developed and underdeveloped countries one of the major health problems is cancer. It is characterized by abnormal development of the tissue in the body parts. Yearly near about 1.8 million peoples is diagnosed with lung cancer. Lung cancer is common in both men and women having exposure to both direct and indirect smoking group [7-9]. Benzimidazole is a lead molecule for most of the biological agent used in the pharmaceutical industry. It consists of a fused benzene ring with heterocyclic aromatic imidazole. The existence of imidazole creates it a resourceful heterocycles with an extensive range of biological activities such as antiulcer (Gastric H^+/K^+-ATPase inhibitors), antihypertensive, anti-inflammatory, anticonvulsant, analgesic, antiprotozoal, anti-trichinellosis, antidiabetic, anti-HIV, anticancer, antimicrobial, antitubercular, antihistaminic, antioxidant, antiparasitic, antiviral, agents, diuretic and DNA binding activities [10-23].

Encouraged by the upstairs findings and in the persistence of our work on coupled mercaptobenzimidazole derivatives, we herein report the synthesis and *in vitro* evaluation of dihydrobenzimidazole thiopyranooxazinone derivatives used as a potent biological agent.

EXPERIMENTAL

Chemicals and solvents of analytical grade required for the synthesis of dihydrobenzimidazole thiopyranooxazinone derivatives were purchased from Sigma-Aldrich and S.D. Fine Chemicals (India). Synthesized compounds were determined for its melting points with the help of precision melting point apparatus and were uncorrected. Completion of the reaction was confirmed by TLC on silica gel-G plates and the spots were visualized in the UV chamber or iodine chamber. IR spectra of intermediates and derivatives compound were recorded by using on KBr pellets on a Jasco FTIR-460 plus spectrophotometer and vibrational frequencies expressed in cm^{-1}. ^1H NMR spectra were recorded on BRUKER 400 MHz spectrometer in deuterated DMSO using tetramethylsilane (TMS) as internal standard and chemical shifts were recorded as δ (ppm).

Synthesis of mercaptobenzimidazole (I): *o*-Phenylenediamine (10.8 g, 0.1 mol) treated with carbon disulfide (7.67 g, 0.1 mol) in the presence of KOH (5.65 g, 0.1 mol), 100 mL of 95% ethanol and 15 mL of water used as a solvent in a round bottom flask was refluxed on a water bath for three hours. After the completion of the reaction, the reaction mixture was allowed to cool and then filtered. After that, 1-1.5 g of activated charcoal was added carefully in the filtrate and refluxed for 10 min on water-bath and then removed the activated charcoal by filtration. The filtrate was treated with 100 mL of warm water at 60-70 ºC for 10 min follwed by the addition of dilute acetic acid into the reaction mixture for acidification with gentle agitation to yield shiny crystals as a product, which is further kept in a refrigerator for 3 h to allow the complete crystallization process. The obtained solid product was separated through Büchner funnel and dried at 40 ºC overnight and recrystallized from the ethanol [24,25]. Yield 73.33%; m.p.: 300-305 ºC; R_f value 0.67, FTIR (KBr, ν_{max}, cm^{-1}): 3155 (N-H), 2993 (C-H, Ar), 1512 (C=C, Ar), 1357 (C-N), 655 (C-S).

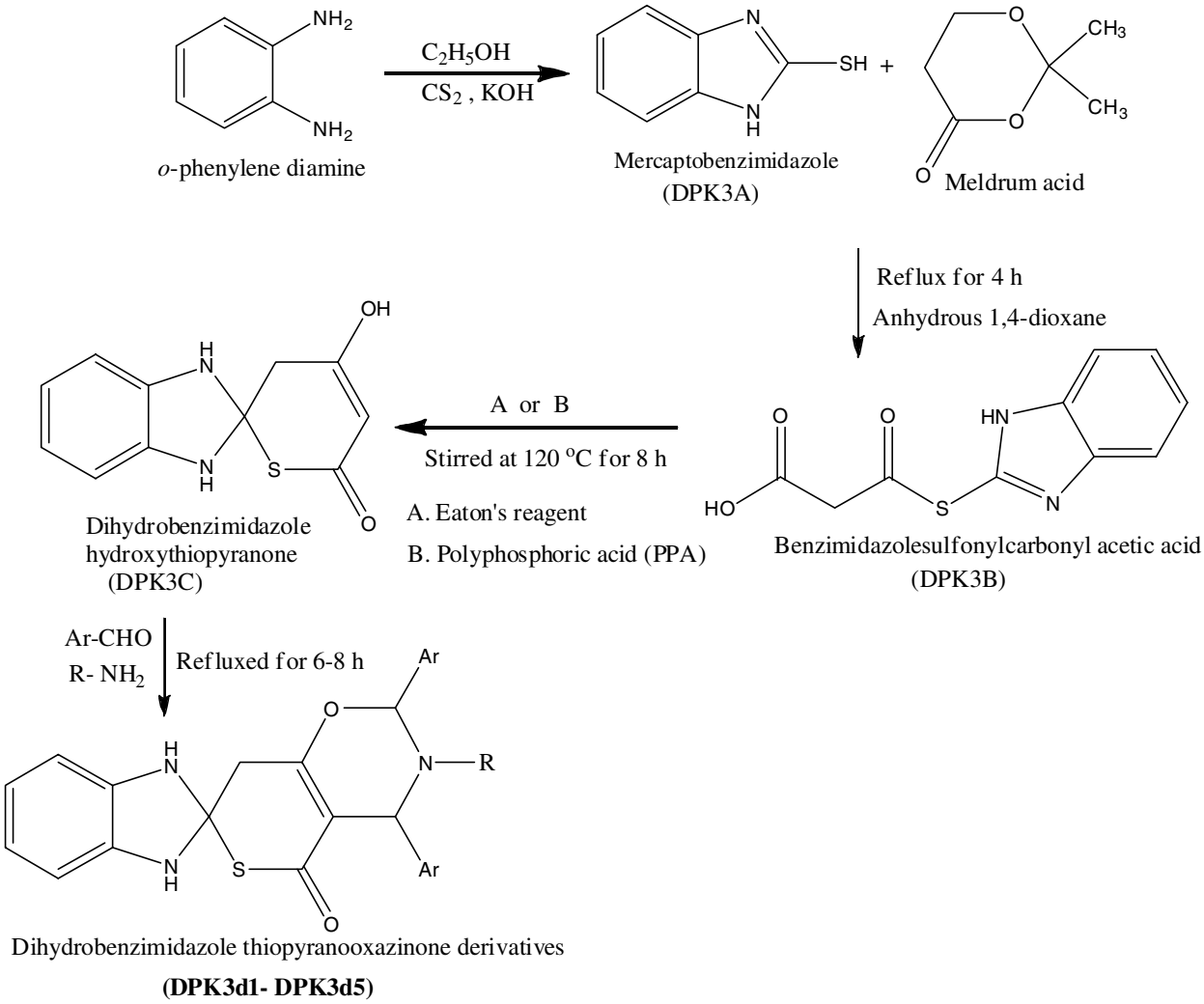
Synthesis of benzimidazolesulfonylcarbonyl acetic acid (II): Mercaptobenzimidazole (150 mg, 0.1 mol) was treated with Meldrum acid (184 mg, 0.1 mol) in the presence of anhydrous 1,4-dioxane (5 mL, 0.1 mol) used as a solvent in a round bottom flask was refluxed on a water bath for 4 h. After the completion of the reaction, the reaction mixture was cooled and filtered. After that, the filtrate was introduced into a separatory funnel and partitioned with ethyl acetate and saturated $NaHCO_3$. From the partitioned solution, the mixture to separate out aqueous layer and acidified at pH 1-2 by adding carefully conc. HCl. Further, the acidified solution extracted several times with dichloromethane. The obtained extract was dried over the magnesium sulfate and concentrate to assume targeted product and recrystallized from the benzene or hexane [24,25]. Yield 57.80%; m.p.: 325-330 ºC, R_f value 0.67; FTIR (KBr, ν_{max}, cm^{-1}): 3155 (N-H), 2993 (C-H, Ar), 2576 (O-H, COOH), 1627 (C=O), 1512 (C=C, Ar) 1357 (C-N), 655 (C-S).

Synthesis of dihydrobenzimidazole hydroxythiopyranone (III): Benzimidazolesulfonylcarbonyl acetic acid (98 mg, o.1 mol) treated with polyphosphoric acid (1 g, 0.1 mol, 116%) or Eaton′s reagent in an Erlenmeyer flask was stirred at 120 ºC for 6-8 h. After the completion of the reaction, the reaction mixture was cooled to room temperature and then added 10 mL of distilled water with vigorous stirring. The obtained precipitate was filtered off and washed again with distilled water. The precipitate was dried and finally recrystallized with absolute ethanol [24,25]. Yield 51.54%; m.p.: 283-287 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3503 (O-H), 3155 (N-H), 2993 (C-H, Ar), 1643 (C=O), 1512 (C=C, Ar), 1357 (C-N), 655 (C-S).

Synthesis of dihydrobenzimidazole thiopyranooxazinone derivatives (DPK3d1-DPK3d5): Dihydrobenzimidazole hydroxythiopyranone (1 g, 0.1 mol) treated with an aromatic aldehyde (1.5 g, 0.1 mol) in the presence ethanol 10 mL in a round bottom flask was refluxed on a water bath for 6-8 h. After that the reaction the mixture was cooled to room temperature; the obtained precipitate was filtered off and dried. The targeted product was recrystallized with absolute ethanol or dimethylformamide (**Scheme-I**).

DPK3d1: Yield 75%; m.p.: 287-289 ºC, R_f value 0.67; FTIR (KBr, ν_{max}, cm^{-1}): 3464 (N-H), 3109 (C-H, Ar), 1627 (C=O), 1450 (C=C, Ar), 1033 (C-N), 964 (C-O-C), 879 (Cl), 709 (C-S).^1H NMR (500 MHz, DMSO-d_6): δ 10.5 (bs, 1H, NH), 7.80 (s, 1H, CH), 7.10-7.60 (m, 3H, Ar-H), 2.50 (s, 1H, CH).

DPK3d2: Yield 80%; m.p.: 294-296 ºC, R_f value 0.72; FTIR (KBr, ν_{max}, cm^{-1}): 3286 (N-H), 3039 (C-H, Ar), 1666 (C=O), 1442 (C=C, Ar),1342 (NO_2, Ar), 1141 (C-N), 1087 (C-O-C), 779 (Cl), 686 (C-S).^1H NMR (500 MHz, DMSO-d_6): δ 12.50 (s, 1H, NH), 10.50 (s, 1H, NH), 7.90-8.00 (s, 1H, CH), 6.50-7.60 (m, 3H, Ar-H), 2.50 (s, 1H, CH).



Scheme-I: Synthesis of dihydrobenzimidazole thiopyranooxazinone derivatives

DPK3d3: Yield 63%; m.p.: 270-272 ºC, R_f value 0.59; FTIR (KBr, ν_{max}, cm^{-1}): 3232 (N-H), 3039 (C-H, Ar), 1666 (C=O), 1442 (C=C, Ar), 1180 (C-O-C), 1149 (C-N), 748 (Cl), 655 (C-S).

DPK3d4: Yield 94%; m.p.: 281-282 ºC, R_f value 0.73; FTIR (KBr, ν_{max}, cm^{-1}): 3255 (N-H), 3093 (C-H, Ar), 1643 (C=O), 1442 (C=C, Ar), 1357 (NO_2, Ar), 1211(C-O-C), 1149 (C-N), 648 (C-S). ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.00 (s, 1H, NH), 10.00 (s, 1H, NH), 8.50 (s, 1H, CH), 6.50-8.20 (m, 3H, Ar-H), 2.50 (s, 1H, CH).

DPK3d5: Yield 88%; m.p.: 283-285 ºC, R_f value 0.70; FTIR (KBr, ν_{max}, cm^{-1}): 3302 (N-H), 3047 (C-H, Ar), 1635 (C=O), 1481 (C=C, Ar), 1365 (NO_2, Ar), 1149 (C-O-C), 1087 (C-N), 601 (C-S). ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.00 (s, 1H, NH), 10.00 (s, 1H, NH), 8.00 (s, 1H, CH), 7.50-7.70 (m, 3H, Ar-H), 2.50 (s, 1H, CH).

***in vitro* Antimicrobial evaluation:** *in vitro* Antimicrobial activity of synthesized compounds were evaluated by the broth dilution method [26-28] against *Escherichia coli* (Gram-negative bacteria), *Bacillus subtilis* (Gram-positive bacteria) and *Aspergillus niger* (fungal strain) using ciprofloxacin and fluconazole as standard antibacterial and antifungal drugs, respectively. All the synthesized derivatives had nine-time dilutions to be done with brain heart infusion (BHI) for minimum inhibitory concen-tration (MIC). Initially, 20 µL of the drugs in the initial tube was added into the 380 µL of BHI broth. For dilution, 200 µL BHI broth was added into the next nine tubes separately. Further, 200 µL broth from the initial tube was transferred to first tube to make 10^{-1} dilution. A 5 µL was taken from the preserved stock cultures of required organisms and was added into 2 µL of BHI broth, then 200 µL of the above culture suspen-sion was added to each sequentially diluted tube. The turbidity of cultures was checked after 24 h of incubation. The minimum inhibitory concentration was the highest dilution of the synth-esized compounds with no visually detectable bacterial or fungal growth.

***in vitro* Antitubercular evaluation:** *in vitro* Antitubercular activity of synthesized compounds were evaluated by Micro-plate Alamar Blue Assay (MABA) against *Mycobacterium tuberculosis* (H37Rv strain) using pyrazinamide, ciprofloxacin and streptomycin as standard drugs [29-31]. This assay proce-dure was non-toxic, and used thermally stable chemical reagent. MABA method exhibited an agreeable connection with BACTEC radiometric and proportional method. During incubation, 200 µL of sterile deionized water was added to all outer perimeter wells of the sterile 96-well plate for minimizing the evaporation of medium in the test wells. The 96-well plate in the columns received 100 µL of the Middlebrook 7H9 broth. A 100 to 0.2

μg/mL of drug concentration were added to the 96-well plate. Parafilm was used to cover and sealed the plates. After that, the plates were incubated at 37 ºC for five days. Freshly prepared mixture of Almar blue reagent (25 μL) and 10% Tween 80 (1:1) was added to the plates and reincubated at 37 ºC for 24 h. The blue colour of the well indicates that no mycobacterial growth and pink colour was counted as mycobacterial growth for synthesized compounds.

***in vitro* Anticancer screening:** *in vitro* Anticancer activity of synthesized compounds were evaluated by sulforhodamine B assay (SRB) method against human lung cancer cell line A-549 using adriamycin as standard drug [32,33]. The RPMI (Roswell park memorial institute medium) 1640 medium (2 mM L-glutamine and 10% fetal bovine serum) useful to grow cell lines. The grown cell lines were inoculated into 96 well microtiter plates in 100 μL at plating densities. The complete inoculation of cell lines, further microtiter plates were at 37 ºC for 24 h with 5% CO_2, 95% air and 100% relative humidity prior to the addition of synthesized compounds. Initially, synthesized compounds at 100 mg/mL solubilized in DMSO, further diluted up to 1 mg/mL and in frozen condition to store for preceding use. During the addition of synthesized compound liquefied frozen concentrate and diluted to various concentration like 100, 200, 400 and 800 μg/mL and make to final dilution up to 10, 20, 40 and 80 μg/mL, respectively. After that, plates were incubated for 48 h as standard conditions and the addition of cold trichloroacetic acid (TCA) with the termination of the assay. *in situ* with fixed cell were adequate addition of 50 μL of cold TCA 30% (w/v) at 4 ºC incubated for 60 min. After that, discarding the supernatant, the microtiter plates five times were washed with water and air-dried. Sulforhodamine B solution (0.4% w/v) in 1% acetic acid solution was added to each of 96 well microtiter plates were incubated at room temperature for 20 min. The completion of the staining process, from the microtiter plates to be recovered the unbound dye and removed the residual dye five times were washed with 1% acetic acid and air-dried. Afterward, the bound stain was eluted with 10 mM trizma base and read the absorbance on a reader plate at a wavelength of 540 nm with 690 nm reference wavelength, finally to calculate the percent growth. The percent growth was calculated as:

$$\text{Growth (\%)} = \frac{\text{Average absorbance of the test wells}}{\text{Average absorbance of the control wells}} \times 100$$

Also, to calculate the percentage growth inhibition of each of the synthesized compounds at different concentration level. Percentage growth inhibition was calculated as:

$$\text{Growth inhibition (\%)} = \frac{T_i}{C} \times 100$$

where, T_i = Test growth in the presence of synthesized compounds at different concentration level and C is the control growth.

***in silico* Target identification and validation for antioxidant activity docking studies using Auto Dock Vina 4.2.6:** Target protein for antioxidant activity: (1CB4 (superoxide dismutase/SOD)).

Preparation of macromolecule and ligands for docking: Autodock Vina 4.2.6 software includes tools for optimization of macromolecule (1CB4 PDB will be taken from www.rcsb.org) as well as ligands, such as assigning atomic charges to make macromolecule more polar, ligand modification through charge and rotatable bonds assignment, calculation of energy contribution of desolvation during ligand binding on a macromolecule, prior assigning of grid maps on the macromolecule surface for interaction with ligands by the auto grid (bind site coordinates X = 16.16, Y = 69.87, Z = 15.33). The above tools improve the flow, efficiency, docking with a new scoring function, effective optimization and multi-threading of molecular docking.

RESULTS AND DISCUSSION

In this work, we have reported synthesis and characterization of substituted benzimidazole derivatives and screened for their *in vitro* antitubercular, antimicrobial and anticancer activities. The purity and homogeneity of substituted benzimidazole derivatives were preliminarily checked by their physical constants. These compounds were characterized by various spectral studies such as IR and ^{1}H NMR for structural elucidation and showed satisfactory results.

***in vitro* Antimicrobial activity:** The results of MIC values of synthesized compounds (μg/mL) against *Bacillus subtilis*, *Escherichia coli* and *Aspergillus niger* are summarized in Table-1. Some dihydrobenzimidazole thiopyranooxazinone derivatives were found to be highly efficient as antimicrobial agents in comparison to the standard drug ciprofloxacin and fluconazole as they represented by their low MIC values compared to standard drugs. Amongst the synthesized compounds **DPK3d1 -DPK3d5** showed significant and potent activity against *A. niger* compared with the standard fluconazole. Whereas, compounds **DPK3d1-DPK3d5** were found to have an average activity against *Bacillus subtilis* and *Escherichia coli* compared with standard ciprofloxacin.

TABLE-1
BIOLOGICAL ACTIVITY AND MIC (μg/mL) VALUES OF SYNTHESIZED COMPOUNDS

Compound	Substituents		Antibacterial activity		Antifungal activity	Antitubercular activity
	Ar-CHO	R-NH_2	*B. subtilis*	*E. coli*	*A. niger*	*M. tuberculosis*
DPK3d1	*p*-Chlorobenzaldehye	Aniline	50	25	1.60	1.60
DPK3d2	*p*-Chlorobenzaldehye	*p*-Nitroaniline	50	100	3.12	12.50
DPK3d3	Benzaldehyde	*m*-Chloroaniline	100	100	6.25	6.25
DPK3d4	Benzaldehyde	*p*-Nitroaniline	50	50	3.12	12.50
DPK3d5	Benzaldehyde	*o*-Nitroaniline	50	100	6.25	6.25
Ciprofloxacin	–	–	2	2	–	3.12
Fluconazole	–	–	–	–	8	–
Pyrazinamide	–	–	–	–	–	3.12
Streptomycin	–	–	–	–	–	6.25

TABLE-2
ANTICANCER ACTIVITY AND GI_{50} VALUES OF SYNTHESIZED COMPOUNDS AGAINST CELL LINE A-549

Compound	Substituents		Control growth (%)			
	Ar-CHO	R-NH_2	10 µg/mL	20 µg/mL	40 µg/mL	80 µg/mL
DPK3d1	*p*-Chlorobenzaldehye	Aniline	89.9	93.1	91.3	104.3
DPK3d2	*p*-Chlorobenzaldehye	*p*-Nitroaniline	93.8	92.2	89.6	99.1
DPK3d3	Benzaldehyde	*m*-Chloroaniline	98.2	104.8	103.1	126.1
DPK3d4	Benzaldehyde	*p*-Nitroaniline	95.4	99.1	99.8	116.0
DPK3d5	Benzaldehyde	*o*-Nitroaniline	100.6	106.2	101.1	104.9
ADR	–	–	20.7	20.6	19.7	34.2

***in vitro* Antitubercular activity:** The results of the MIC values of synthesized compounds against *Mycobacterium tuberculosis* (H37Rv) are summarized in Table-1. It is evident that among the five compounds out of three compounds such as **DPK3d1**, **DPK3d3** and **DPK3d5** showed potent antitubercular activity compared with standard antitubercular drugs such as pyrazinamide, ciprofloxacin and streptomycin, rest of the synthesized compounds (**DPK3d2**, **DPK3d4**) had shown moderate to good antitubercular activity.

***in vitro* Anticancer activity:** The results of GI_{50} values of synthesized compounds against human lung cancer line A-459 are summarized in Table-2. Amongst the synthesized compounds **DPK3d1**, **DPK3d3** and **DPK3d5** showed potent anticancer activity compared to standard adriamycin.

Structure activity relationship: From the comparison of antimicrobial, antitubercular and anticancer activities of synthesized dihydrobenzimidazole thiopyranooxazinone derivatives, the following SAR may be assumed.

1. Antimicrobial activity of the synthesized dihydrobenzimidazole thiopyranooxazinone compared to the standard drug ciprofloxacin and fluconazole concluded that there should be slight structural modifications to develop affinity of the drug to the binding of a molecule to the target site.

2. Antitubercular activity of the synthesized dihydrobenzimidazole thiopyranooxazinone derivatives compared to standard drugs such as pyrazinamide, ciprofloxacin and streptomycin pointed that the synthesized compounds have a very good interaction with the target sites and have need of supplementary *in vivo* studies to confirm the antitubercular activity.

3. Anticancer activity of the synthesized dihydrobenzimidazole thiopyranooxazinone derivatives compared to the standard drugs such as adriamycin may draw attention that the synthesized compounds have a very good interaction with target sites however, *in vivo* studies are required to confirm the anticancer activity.

4. The structure activity relationship amongst the dihydrobenzimidazole thiopyranooxazinone derivatives outcomes are summarized as follows:

[A] at position R_1 & R_2: (i) Presence of benzene ring essential for antimicrobial, antitubercular, anticancer activities, and (ii) substitution of Cl group at *para*-position shows potent antimicrobial activity and moderate to good antitubercular, anticancer activities.

[B] at position R_3: (i) Presence of benzene ring essential for antimicrobial, antitubercular, anticancer activities; (ii) at *para*-position substitution of Cl group shows potent antimicrobial activity and moderate to good antitubercular, anticancer activities; and (iii) at *ortho*-position substitution of NO_2 and Cl group shows potent antitubercular, anticancer activities and moderate to good antimicrobial activity.

Docking studies: In present work, the docking or binding-free energy as shown in Fig. 1, which reflects the binding affinity of five ligands. The above docking studies signify the fact that all the ligands show the highest binding affinity range -6.7 to -8.7 (Table-3). Therefore, all the selected ligands, which showed better docking energy with respective 1CB4 PDB of superoxide dismutase.

TABLE-3
PREDICTED BEST INTERACTION OF USING AUTODOCK 4.2.6

Sample code	Binding energy (kcal/mol)
DPK3d1	-6.7
DPK3d2	-8.3
DPK3d3	-7.1
DPK3d4	-8.4
DPK3d5	-8.7

Conclusion

Dihydrobenzimidazole thiopyranooxazinone derivatives were efficiently synthesized and screened for their *in vitro* antimicrobial, antitubercular and anticancer activities. Amongst the synthesized compounds **DPK3d1-DPK3d5** showed significant and potent activity against *Aspergillus niger* compared with the standard fluconazole. Derivatives such as **DPK3d1**, **DPK3d3** and **DPK3d5** shown potent antitubercular activity against *Mycobacterium tuberculosis* compared with standard pyrazinamide, ciprofloxacin and streptomycin. Also, derivatives such as **DPK3d1**, **DPK3d3** and **DPK3d5** showed potent anticancer activity compared with the standard adriamycin. *In silico* molecular docking studies shown that all the ligands highest binding affinity range -6.7 to -8.7 for selected 1CB4 PDB of superoxide dismutase, which recognized that ligands having antioxidant activity.

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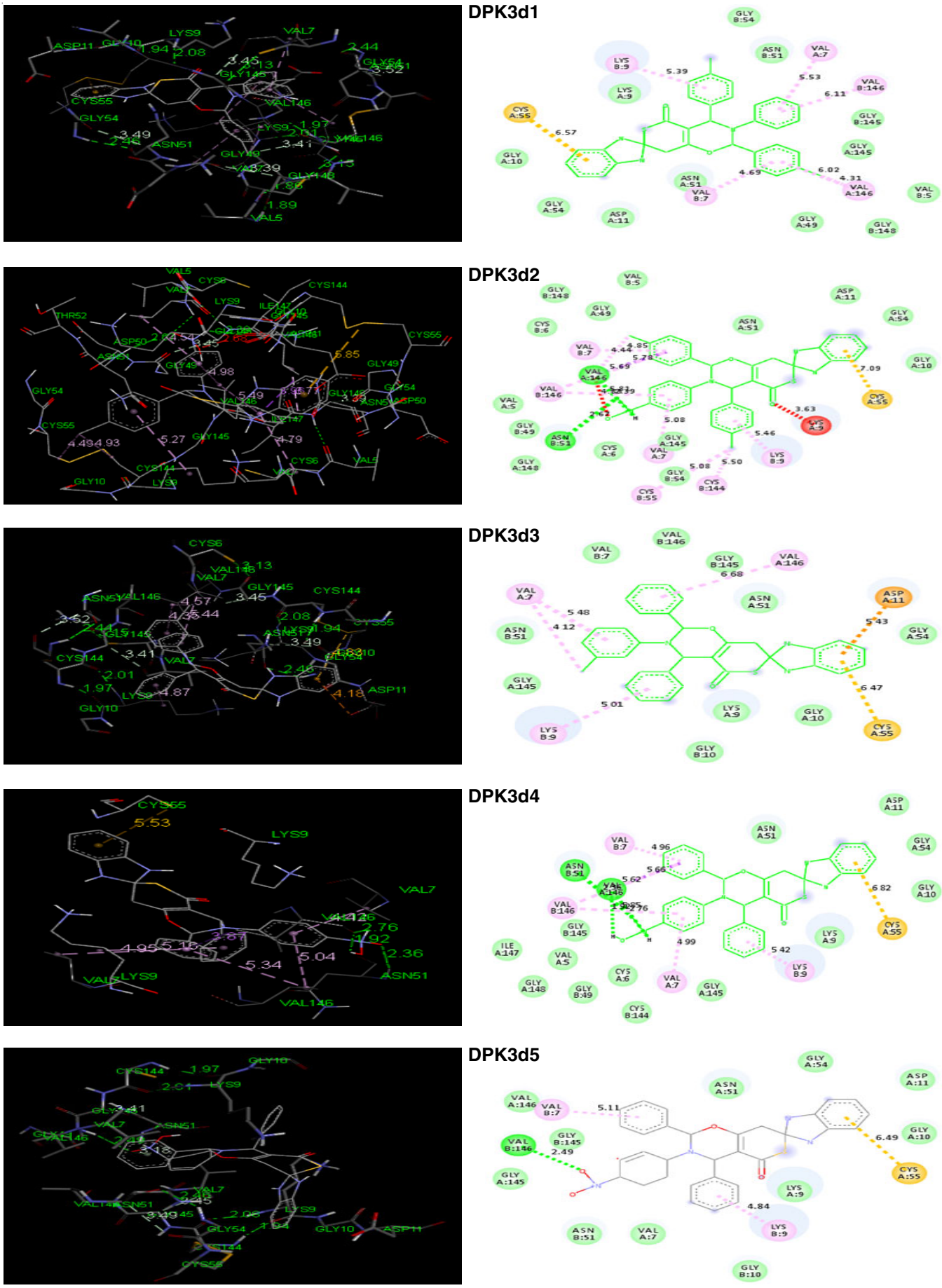

Fig. 1. Docking Pose of 1CB4 Superoxide Dismutase/ SOD with dihydrobenzimidazole thiopyranooxazinone derivatives

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