Synthesis, cytotoxic and urease inhibitory activities of some novel isatin-derived bis-Schiff bases and their copper(II) complexes

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Material and methods

Biological assays

Cytotoxicity assay

Cell lines and cultures

Lung carcinoma (H-157), (ATCC CRL-5802) and African green monkey kidney normal cell line (Vero), (ATCC CCL-81) were kept in RPMI-1640, having heat-inactivated fetal bovine serum (10%) glutamine (2 mM), pyruvate (1 mM), 100 U/mL penicillin and 100 µg/mL streptomycin, in T-75 cm² sterile tissue culture flasks in a 5% CO₂ incubator at 37 °C.¹ For experiment, 96-well plates were used for growing H-157 and Vero cells by inoculating \(5 \times 10^4\) cells per 100 µL per well and plates were incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO₂. Within 24 h, a uniform monolayer was formed which was used for experiments.

Cytotoxicity analysis by sulforhodamine B (SRB) assay

To perform cytotoxicity assay, with H-157 and Vero cells, a previously described method by Skehan et al.² was adapted with little modifications.³ Briefly, cells were cultured in different 96
well plates for 24 h. The compounds in different concentrations were inoculated in test wells. Furthermore, positive control vincristine was prepared in DMSO. The well containing culture media with cells having no compound or drug was taken as blank. Vero cells were treated at 100 µM test compounds to check the toxicity against normal cell lines. The plates were then incubated for 48 h. After that, cells were fixed with 50 µL of 50% ice cold trichloroacetic acid solution (TCA) and plates were incubated at 4 °C for 1 h. Subsequently, plates were washed five times with phosphate-buffered saline (PBS) and air dried. Fixed cells were further treated with 0.4% w/v sulforhodamine B dye (prepared in 1% acetic acid solution) and left at room temperature for 30 min. After that the plates were rinsed with 1% acetic acid solution and allowed to dry. In order to solubilize the dye, the dried plates were treated with 10 mM Tris base solution for 10 min at room temperature. The absorbance was measured at 490 nm, subtracting the background measurement at 630 nm. All the experiments were repeated at least three times. Results reported are mean of three independent experiments (± SEM) and expressed as percent inhibitions calculated by the formula:

\[
\text{Inhibition (\%) = 100 - \left(\frac{\text{absorbance of the test compounds}}{\text{absorbance of the control}}\right) \times 100}
\]

IC\textsubscript{50} values of potential inhibitors (≥50%) were determined with the help of the Graph Pad prism 5.0 Software Inc., San Diego, California, USA.

**Urease inhibition assay**

Urease inhibition activity of the synthesized compounds was determined by indophenol method with little modification. Reaction mixtures comprising 40 µL of buffer (100 mmol/L urea, 0.01 mol/L K\textsubscript{2}HPO\textsubscript{4}, 1 mmol/ L EDTA and 0.01 mol/L LiCl\textsubscript{2}, pH 8.2) and 10 µL of enzyme (5 U/mL) were incubated with 10 µL of test compounds (1 mM) and thiourea at 37 °C for 30 min in 96-well
plates. Urease inhibitory activity was calculated by indophenol method based on the production of ammonia. The phenol reagent (40 μL, 1 %, w/v phenol, 0.005 %, w/v sodium nitroprusside) and alkali reagent (40 μL, 0.5 %, w/v NaOH, 0.1 % active chloride NaOCl) were added to each well and after 10 min of incubation at 37 °C, the absorbance was measured at 630 nm using a microplate reader (Bio-TekELx 800™, Instruments, Inc. USA). All the experiments were performed in triplicate. Following equation was used for calculation of percentage inhibition.

\[
\text{Percentage Inhibition} = \left(1 - \frac{\text{Experimental Absorbance}}{\text{Control Absorbance}}\right) \times 100
\]

Thiourea was used as the standard inhibitor. In order to calculate IC\textsubscript{50} values, different concentrations of synthesized compounds and standard were assayed at the same reaction conditions. The results were analyzed using PRISM 5.0 (GraphPad, San Diego, California, USA).

**Docking protocols**

**Structure selection and preparation**

Molecular docking studies were conducted to investigate putative interactions of the compounds in complex with the urease enzyme. In order to perform efficient docking studies, the crystallographic structure of Jack bean urease (PDB ID: 4H9M) was obtained from the RSCB PDB database\textsuperscript{7} and prepared for the docking. Prior to experiments, the structures of the enzyme and the compounds were prepared as follows. The enzyme structure was protonated with the Protonate 3D\textsuperscript{8} algorithm implemented in the molecular modeling tool MOE.\textsuperscript{9} The structure was energy minimized using Amber99 force field including all crystallographic solvent molecules. The backbone atoms were restrained with a small force in order to avoid collapse of the binding pockets during energy minimization calculations. After minimization, the co-crystallized ligands and solvent molecules were removed. The bound ligand acetohydroxamic acid and crystallographic
water molecules were removed from the structure and hypothetical hydrogen atoms were added to the X-ray structure in standard geometries with the MOE.

**Compounds preparation**

The 3D structural coordinates of the compounds were generated for all the compounds using MOE followed by assignment of protonation and ionization states in physiological pH range by using the “wash” module. Afterwards, the compounds' structures were energy minimized with the MMFF94x force field for docking studies.

**Docking studies**

For the docking studies, AutoDock Tools\textsuperscript{10} was used to add atomic partial charges to the protein structure and the ligands. The point charge of the two catalytic nickel cations was manually set to +2. Gasteiger charges were calculated using AutoDock Tools.\textsuperscript{10} The newly synthesized ligands (Schiff bases) were docked into the active sites of urease using the Lamarckian Genetic Algorithm in AutoDock 4.2 with rigid protein structure and fully flexible ligands. Possible binding modes of the Schiff bases were explored by visual inspection of the resulting docking poses. The Discovery Studio 4.0 Visualizer was used for visualizing the results.\textsuperscript{11}

**Results and Discussion**

**Chemistry of the copper(II) complexes 5a-g**
Scheme S1. Synthesis of title Schiff base Cu(II) complexes 5a-g.

R:
5a = H
5b = Br
5c = F
5d = Cl
5e = CH₃
5f = SO₃H
5g = NO₂
Figure S1. The proposed structural formulae of metal complexes 5a-g.
Raman spectra of some representative compounds

Figure S2. Raman spectrum of 5c [Cu(L₃)₂Cl₂]
Figure S3. Raman spectrum of 5d $[\text{Cu(L}_4\text{)}_2\text{Cl}_2]$
Figure S4. Raman spectrum of 5e [Cu(L₅)₂Cl₂]
Figure S5. Raman spectrum of 5g [Cu(L7)2Cl2]
Figure S6. Overlaid TG curves of Cu(II) complexes 5a-g.
Figure S7. Overlaid DTG curves of Cu(II) complexes 5a-g.
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