Novel Schiff base copper complexes of quinoline-2 carboxaldehyde as proteasome inhibitors in human prostate cancer cells

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Running title: Novel Schiff base copper complexes as proteasome inhibitors

Supporting Information

Contents of SI: Contains experimental details for the determination of biological activity, spectral data, and elemental analysis data for all new compounds.

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Instrumentation

Elemental analyses were carried out on Hosley C H N analyzer. IR spectra were recorded in nujol-mull in the range 4000-500 cm^{-1} on Shimadzu FTIR-8400 infrared spectrophotometer, while electronic spectra were recorded on a Spectronic Genesys-2 spectrophotometer using matched pair of $1cm^3$ quartz cells in the range 250-1100 nm. Conductivity of the metal complex was measured on a conductivity instrument EQ-664 from EQUIP-TRONICS. The instrument was calibrated by using 0.1 M KCl in the solvent prior to use. The EPR spectra of the compounds were recorded in DMSO at 77K on a Varian X-band spectrometer. TCNE compound was used as a field marker. Magnetic susceptibilities of the metal complexes were measured at 298 K on a Faraday balance with the field strength of 7000 G using Hg [Co $(SCN)_4$]. Cyclic voltammetric measurements were made in dimethylsulfoxide (DMSO) solvent on a BAS CV-27 instrument with an XY recorder using Pt disc as the working electrode against SCE and Pt wire as an auxiliary electrode with Tetraethyl ammonium perchlorate (TEAP) as the supporting electrolyte.

All reagents were of analytical-reagent (AR) grade and unless otherwise noted, all solvents, chemicals and reagents were obtained commercially and used without purification. Benzoyl hydrazide was prepared according to the method described in the literature [1] and thiosemicarbazide hydrochloride was made following a procedure established in our laboratory. [2] Solvents employed were purified by standard protocols prior to their use. [3]

Experimental Methods

Chemistry

*Synthesis of quinoline-2-carboxaldehyde Schiff bases (**1**, **3**, **5** and **7**)*

The Schiff base ligands **1**, **3**, **5** and **7** (Scheme 1) were synthesized by mixing equimolar amounts of quinoline-2 carboxaldehyde with thiosemicarbazide hydrochloride, benzoyl hydrazide, isonicotinoyl hydrazide and salicylic hydrazide, respectively, in methanolic solvent and maintaining the reaction mixture at reflux temperature for 1hr. The products obtained were filtered off, recrystallized from (1:1) DMF-methanol and finally dried in vacuum desiccators over anhydrous $CaCl_2$.

*Synthesis of copper complexes (**2**, **4**, **6** and **8**)*

The copper (II) complexes of **1**, **3**, **5** and **7** were synthesized by mixing equimolar amounts of the methanolic solutions of ligands and $CuCl_2 \cdot 2H_2O$. The resulting mixture was refluxed at room temperature for 1h. The precipitates formed were removed by filtration, washed with the methanol solvent and dried in a vacuum over anhydrous $CaCl_2$. The analytical elemental data for the copper complexes (**2**, **4**, **6** and **8**) of quinoline-2-carboxaldehyde are given in Table 1 under Supporting Information.

Biology

Cell Culture

Two cancer cell lines, PC-3 and LNCaP prostate cancer cell lines were chosen (American Type Cell Culture Collection, Manassas, VA). PC-3 and LNCaP were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37^oC.

Reagents for proteasome assay

Purified rabbit 20S proteasome, fluorogenic proteasomal chymotrypsin-like peptide substrate Suc-Leu-Leu-Val-Tyr-AMC were obtained from Calbiochem Inc. (San Diego, CA). Another fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity was from BIOMOL International LP (Plymouth Meeting, PA).

Cell growth inhibition assay by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Two cancer cell lines were seeded in 96-well culture plates. After 24 hours of incubation, PC-3 and LNCaP cells were treated with 1, 5, 10, 15, 20, μM of compounds **1** to **8** (**FPA-136 to 143**) for 72 h. The number of viable cells remaining after an appropriate treatment was determined by the MTT assay. Briefly cells were plated (4,000 cells/well per 0.2 ml RPMI 1640 medium) in 96-well micro titer plates and incubated overnight. The test compound was then added to each well at a fixed concentration in quadruplicate wells. After treatment, cells were incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL, Sigma) at 37°C for 2 hours and then with isopropyl alcohol at room temperature for 1 hour. The spectroscopic absorbance of the samples was determined by ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 595 nm as described previously. [4] For IC_{50} values, the mean and standard deviation (SD) of relative growth was graphed versus log concentration of compound. The IC_{50} was calculated by sigmoidal dose response curve using Prism software (GraphPad, San Diego, CA).

Histone/DNA ELISA for detecting apoptosis

Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis according to the protocol supplied by the manufacturer. Briefly, PC-3 and LNCaP cells were treated with two concentrations (one at IC_{50} and the other above IC_{50} values) of the compounds **1** to **8**. After treatment, the cytoplasmic histone / DNA fragments from cancer cells with different treatments were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by ULTRA Multifunctional Microplate Reader (TECAN) at 405 nm as described previously. [4]

Western blot analysis

Cell lysates (50 μg) were subject to SDS–PAGE and then transferred to a nitrocellulose membrane, followed by incubation with primary antibodies and subsequently with secondary antibodies and the signal was visualized via the enhanced chemiluminescence (ECL) kit (Amersham Biosciences). The ECL Western blot analysis was also performed using specific antibodies to ubiquitin and PARP as described previously. [4] Proteasome inhibition was measured as accumulation of ubiquitinated proteins and PARP cleavage which served as a marker for apoptosis.

In vitro proteasome activity assays

The chymotrypsin-like activity of proteasome was measured as previously described. [5] Briefly, purified 20S rabbit proteasome (0.1 μg) or human prostate cancer LNCaP cell extract (10μg) was incubated in 100μl of assay buffer (50 mmol/L Tris–HCl, pH 7.5), with or

without different concentrations of each tested reagent and 20 μmol/L fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 2 h at 37° C. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor3^{TM} multilabel counter with an excitation filter of 355 nm and an emission filter of 460 nm.

Inhibition of the proteasome activity in intact tumor cells

To measure the inhibition of proteasome activity in living tumor cells, 100 μl of LNCaP (1×10^4 cells/well) cells were cultured in a 96-well plate. These cells were treated with or without various concentrations of tested reagents for 18 h, followed by an additional incubation for 2 hr with the fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity. Afterwards, production of hydrolyzed AMC groups was measured using the same plate reader and conditions mentioned above.

Hydrogen Peroxide (H_2O_2) detection

LNCaP cells were treated with two concentrations (IC_{50} and one above IC_{50}) of Cu alone, **1**, solution mixture of **1** and $CuCl_2$ and **2** for upto 24 hr, followed by assaying the presence of H_2O_2 using Amplex® Red Hydrogen Peroxide Kit (Molecular Probes) in accordance with the manufacturer's directions.

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Table 1

Compound	Elemental Analysis					μ eff. BM	EPR g// g⊥ (gauss)	ΛM Ohm-l cm2 mol-l	UV-Vis (nm) b	E*1/2* (V)
	%C	%H	%N	%S	M%					
1 (FPA 136)	57.37 (57.49)	4.38 (4.54)	24.33 (23.92)	13.92 (12.95)	-	-	-	-	305,320	-
3 (FPA 138)	74.17 (74.13)	4.76 (4.86)	15.26 (14.73)	- -	-	-	-	-	310,330	-
5(FPA 140)	69.55 (68.33)	4.38 (4.76)	20.28 (19.63)	-	-	-	-	-	308,325	-
7 (FPA 142)	70.09 (69.63)	4.50 (4.37)	14.42 (13.83)	-	-	-	-	-	320	-
2 (FPA 137	36.26 (36.54)	2.74 (2.45)	15.38 (14.91)	17.44 (16.93)	13.91 (13.71)	1.65	2.28 2.06	31	300,325 450,625	+0.37 r
4 (FPA 139)	49.87 (48.95)	3.17 (2.94)	10.26 (9.45)	-	15.52 (14.83)	1.68	2.29 2.08	28	300,335 410,640	+0.36 r
6(FPA 141)	46.82 (45.90)	2.92 (2.51)	13.65 (12.91)	-	15.48 (14.93)	1.70	2.30 2.11	22	300,330 420,635	+0.33 r
8 (FPA 143)	48.00 (47.51)	3.58 (3.01)	9.88 (8.93)	-	14.94 (14.45)	1.68	2.29 2.08	29	340,405,650	+0.34 r

Table 2

Compound	ν(C=O)	ν(C=O) amide ν(C=N)	ν(C=N) pyridine	ν(C=S)	ν(NH)
I	1709	-	-	-	-
1 (FPA 136)	-	1602	1583	1116	3273,3399
3 (FPA 138)	-	1657	1558	-	3192,3395
5(FPA 140)	-	1663	1558	-	3188,3407
7 (FPA 142)	-	1632	1547	-	3183,3385
2 (FPA 137	-	1522	1450	1091	3265,3381
4 (FPA 139)	-	1498	1431	-	3183,3381
6(FPA 141)	-	1501	1417	-	3175,3491
8 (FPA 143)	-	1500	1456	-	3172,3375

Figure 1

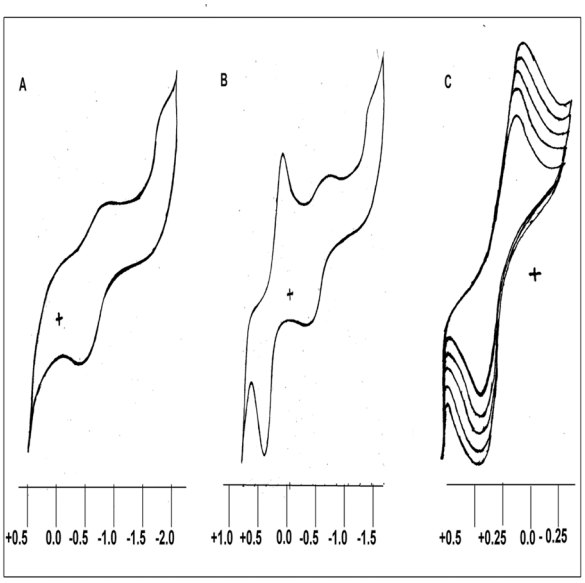

Figure 1: Cyclic voltammetry scan of (a) **1**; (b) **2** and (c) scan rate dependence of **2**.

Figure 2

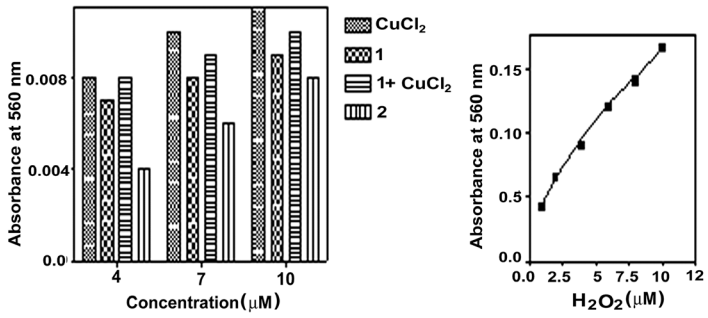

Figure 2: (A) Plot of absorbance at 506 nm indicating induction of H_2O_2 versus concentration of Cu alone, **1**, mixture of **1** and $CuCl_2$ and **2** (4, 7 and 10 μM), respectively. Treatment of LNCaP cells with 10 μM copper complex **2** shows almost 2-fold lower absorbance (B) as compared to standard H_2O_2 curve where 10 μM of H_2O_2 shows strong absorbance at 560 nm indicating high levels of H_2O_2 in LNCaP (untreated control) cells.