Novel Schiff Base Copper Complexes of Quinoline-2 Carboxaldehyde as Proteasome Inhibitors in Human Prostate Cancer Cells

Shreelekha Adsule,[†] Vivek Barve,[‡] Di Chen,[§] Fakhara Ahmed,[†] Q. Ping Dou,[§] Subhash Padhye,[†,‡] and Fazlul H. Sarkar*,[†]

The Prevention Program and Department of Pathology, Barbara Ann Karmanos Cancer Institute, Wayne State University, School of Medicine, 9374 Scott Hall, 540 East Canfield Avenue, Detroit, Michigan 48201, and Department of Chemistry, University of Pune, Pune 411007, India

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We report the synthesis of novel 1:1 Schiff base copper complexes of quinoline-2-carboxaldehyde showing dose-dependent, antiproliferative, and proapoptotic activity in PC-3 and LNCaP prostate cancer cells. We found that quinoline thiosemicarbazone **2** (FPA-137) was the most potent and inhibited proteosome activity in intact human prostate cancer PC-3 and LNCaP cells (IC_{50} of 4 and 3.2 μM, respectively) compared to clioquinol and pyrrolidine dithiocarbamate (IC_{50} of 10 and 20 μM), supporting the novelty of **2**.

Introduction

The ubiquitin−proteasome pathway has been extensively studied in human cancers, which is known to play an important role in regulating cell proliferation and cell death.[1,2] It has been well-established that through a tight regulation of cell growth inducers and growth inhibitors, an optimum balance of cell proliferation and cell death can be achieved. In cancer cells, however, an altered balance between cell growth inducers and inhibitors leads to disruption of the cell cycle, causing deregulated growth and inhibition of intrinsic apoptotic cell death pathways. Thus, proteasome inhibition is a desirable target for controlling the aberrant growth of tumor cells.

Proteasome inhibitors are known to induce cell death rapidly and selectively in oncogene-transformed but not normal or untransformed cells. They are also able to trigger apoptosis in human cancer cells that are resistant to chemotherapy.[3,4] Many natural and synthetic inhibitors of proteasome have been developed, which include lactacystin,[5] threonine benzyl ester macrocycle (TMC95),[6] peptide aldehydes,[7] peptide boronates,[8] peptide inyl sulfones,[9] epoxyketones,[10] peptide-α-keto aldehydes[11] and α-ketoamides, 5-methoxy-1-indanone-3-acetic peptide derivatives,[12] and bivalent inhibitors.[13] Z-Leu-Leu-Leu-aldehyde (MG 132) was one of the first synthetic inhibitors to be described and used in the inhibition of proteasome.[14] Most of them have the amide structural motif necessary for specific recognition by the proteasome site. The major outcome from the work by Dou et al. has shown that neither 8-hydroxyquinoline nor copper(II) chloride alone can inhibit proteasome or induce apoptosis but the copper complex of 8-hydroxyquinoline can potently and selectively inhibit the chymotrypsin-like activity of the proteasome in vitro and in vivo. [15] In our earlier studies, we have shown that certain heterocyclic compounds such as clioquinol and pyrrolidine dithiocarbamate act as potent antitumor compounds by binding to endogenous copper in prostate and breast cancer cells.[16] We have proposed that highly elevated levels of copper can be tumor-specific and use of copper chelators might be one of the useful strategies for cancer therapies. The levels of copper seem to vary in in vitro cultures from low to trace levels, whereas in in vivo situations the serum copper levels are as high as 2 μg/100 mL (equivalent to 0.3 μM) and in malignant prostates as high as 124 μg/100 mL (equivalent to 19.5 μM).[17] The role of copper metal in the ubiquitin−proteasome pathway is as yet undetermined. Although copper alone has been known to inhibit the activity of purified proteasome[15] and copper complexes are able to inhibit the cellular proteasome activity, the exact mechanism remains to be explored.

The quinoline scaffold having a formyl/acetyl group adjacent to a heterocyclic nitrogen can be easily appended with pharmacophores bearing amino groups to yield Schiff base compounds with a wide range of transition metals such as copper, although their potential of arresting growth of cancer cells remained unexplored.[18] In the present study, we describe synthesis and characterization of four such Schiff bases of quinoline-2-carboxaldehyde **1**, **3**, **5**, and **7** (FPA 136,138, 140, and 142) and their copper complexes **2**, **4**, **6**, and **8** (FPA 137, 139, 141, and 143). The cytotoxic effects of quinoline derivatives **1**−**8** were tested in prostate cancer cell lines PC-3 and LNCaP, and the apoptotic cell death was measured by histone/DNA enzyme-linked-immunosorbent assay (ELISA). We further investigated the in vitro effects of these compounds in LNCaP cells and observed accumulation of ubiquitinated proteins and the apoptosis-specific poly ADP-ribose polymerase (PARP) cleavage after 24 h. On the basis of our results, we propose that the hydrophilic thiocarbonyl side chain may confer antiproliferative activity of quinoline−copper complexes, which could inhibit the ubiquitin−proteasome pathway in human cancers.

Experimental Section

Chemistry. The novel complexes **1**, **3**, **5**, and **7** were obtained in satisfactory yields by reacting the parent ligand quinoline-2-carboxaldehyde with thiosemicarbazone and various hydrazines such as benzoyl, nicotyl, and salicyl hydrazides in refluxing methanol. Further, the copper complexes were obtained by heating 1 equiv of the free ligands and $CuCl_2$ in methanolic solution at 40 °C. Overall, the synthetic strategy is summarized in Scheme 1.

Compositional Studies. The analytical and spectroscopic data on all synthesized ligands and their metal complexes are summarized in Table 1 in Supporting Information, which indicate 1:1 stoichiometry for all copper complexes. The metal complexes are insoluble in nonpolar organic solvents but dissolve in polar organic solvent like DMSO. Their molar conductivities in DMSO indicate the nonelectrolyte nature for these compounds.[19] On the basis of the microanalytical data, it is concluded that the obtained Cu(II)

* To whom correspondence should be addressed. Phone: 313-576-8327. Fax: 313-576-8389. E-mail: fsarkar@med.wayne.edu.
[†] Department of Pathology, Wayne State University, School of Medicine.
[‡] University of Pune.
[§] The Prevention Program and Department of Pathology, Wayne State University, School of Medicine.

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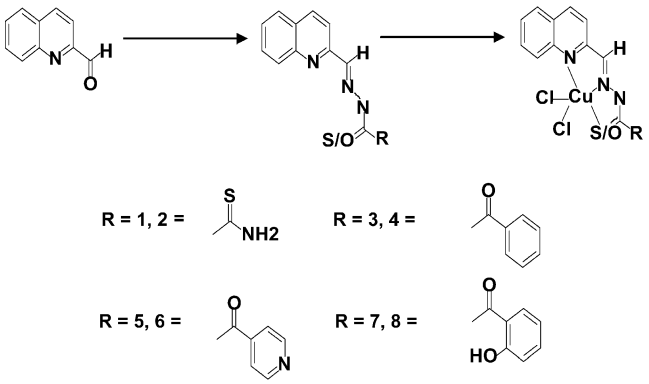
Scheme 1. Synthesis of Quinoline-2-carboxaldehyde Derivatives **1**, **3**, **5**, and **7** and Their Copper Complexes **2**, **4**, **6**, and **8**



complexes exist in a monoaligned fashion that requires a ratio of 1:1:1 for ligand, metal ion, and counterion such as chloride.

Spectroscopy and Magnetism. The electronic spectra of the ligands **1**, **3**, **5**, and **7** in DMSO solvent exhibit intense bands in the range 300−350 nm, which are assigned n → π^* transitions (Table 1 in Supporting Information).[20] All copper compounds exhibit a band around 600−700 nm suggestive of square-pyramidal geometry around the central metal ion with $d_{x^2-y^2}$ as the ground state.[21] The absorption at 450 nm observed for the compound **2** is ascribed to the S → Cu(II) charge-transfer band,[22] while the absorption in the region 400−430 nm is due to the oxygen to copper charge-transfer transition.[23]

The observed magnetic moments for the copper complexes are in the range 1.6−1.7 μ_B, which is characteristic of monomeric copper complexes. The slightly lowered magnetic moment (∼1.65 μ_B) observed in the case of metal complex **2** is probably due to the covalent nature of the metal−sulfur bond (Table 1 in Supporting Information).[24] The low-temperature EPR spectra of the copper complexes were recorded in DMSO glass at 77 K. The spectrum of **2** display four-line copper hyperfine lines and yield a relation $g_{\|} > g_{\perp} > 2.0$ characteristic of monomeric copper complex (Table 1 in Supporting Information). A similar feature has been observed in the spectrum of other copper complexes. Kivelson and Nieman have pointed out that compounds having $g_{\|} \geq 2.3$ are ionic in nature while those with $g_{\|} < 2.3$ are covalent in character. The $g_{\|}$ values for the present series of complexes are higher than $g = 2.0$, revealing the square-pyramidal geometry.[25]

The infrared spectrum of the parent compound (**I**) quinoline-2-carboxaldehyde has a band at 1709 cm^{-1} (Table 2 in Supporting Information) due to the carbonyl group. On the other hand, the band at 1583 cm^{-1} is due to the pyridine nitrogen atom. On condensation with the thiosemicarbazide side chain, the carbonyl absorption is replaced by a new imine band at 1602 cm^{-1},[26] with two additional bands around 3273 and 3399 cm^{-1} due to the asymmetric and symmetric stretches of the terminal amino group.[27] The thiocarbonyl absorption in **1** is located at 1116 cm^{-1} for this compound, while in case of the hydrazone derivatives (**3**, **5**, and **7**), the stretching NH vibration is observed between 3200 and 3400 cm^{-1}. The amide carbonyl frequencies for the compounds can be seen around 1600-1665 m^{-1}.[28] During metal complexation the Schiff base ligands (**1**, **3**, **5**, and **7**) behave as a neutral, tridentate moiety forming two five-member chelate rings around the central metal ion through donor atoms. The imino frequency (C═N) and thiocarbonyl sulfur (C═S) for ligand **1** observed at 1602 and 1116 cm^{-1} were found to be shifted to lower wavenumbers upon complexation (1522 and 1091 cm^{-1}) indicating their involvement in metal coordination. Similarly, such an observation has been observed in the case of the ligands (**3**, **5**, and **7**), as shown in Table 2 in Supporting Information. The absence of a band around 2600−2500 cm^{-1} in **1** indicates the presence of a thione form for this compound.[29]

Cyclic Voltammetry. Under the experimental conditions, the Schiff base ligands (**1**, **3**, **5**, and **7**) exhibit an irreversible reduction peak around −1.00 V, which is due to reduction of the azomethine (C═N) function.[30] An additional quasi-reversible peak centered between −0.55 to −0.75 V is assigned to reduction of aroylhydrazone moiety.[31] The corresponding copper complexes (**2**, **4**, **6**, and **8**) exhibit a reversible Cu(II)/Cu(I) redox couple centered in the range +0.33 to +0.37 V (Figure 1 in Supporting Information), indicating a facile reduction. The ipa/ipc ratios for all copper complexes fall in the range 0.8−0.94, indicating that the copper redox couples are 75−95% reversible.

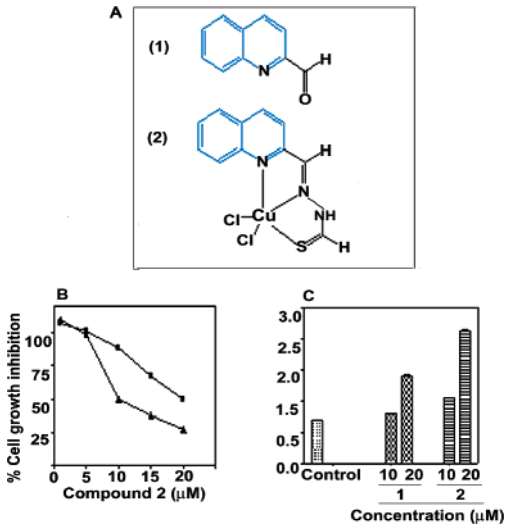


Figure 1. (A) Chemical structures of compounds **1** and **2**. (B) Plot of cell growth inhibition in LNCaP cells after treatment with **1** and **2** for 72 h. (C) Plot showing ELISA apoptosis assay in LNCaP cells (control, 10 and 20 μM **1** and **2**, respectively) for 72 h.

Table 1. IC_{50} Values of Compounds **1**−**8** by MTT Assay in Prostate Cancer Cell Lines PC-3 and LNCaP

	IC_{50} (μM)	
compd	PC-3	LNCaP
1	16	21
2	5	7
3	20	25
4	NE[a]	>50
5	NE[a]	NE[a]
6	20	>50
7	20	31
8	30	35

[a] NE: not effective.

Biological Results and Discussion

Compounds **1**−**8** shown in the Scheme 1 were tested for their cell growth inhibitory effect in PC-3 and LNCaP prostate cancer cell lines. The IC_{50} values by MTT assay in PC-3 and LNCaP cells are given in Table 1. Interestingly, the most effective derivative was found to be **2**, the copper complex of the Schiff base quinoline thiosemicarbazone, which showed high cell kill in LNCaP and PC-3 cell at IC_{50} of 5 and 7 μM after 72 h of treatment. The chemical structures of **1** and **2** are shown in Figure 1A. A dose-dependent cell growth inhibition of LNCaP cells after treatment with **1** and its copper complex **2** at 72 h was observed (Figure 1B).

The isonicotyl hydrazone derivative of quinoline, **5**, was ineffective in all the cell lines tested, whereas the copper complex **6** showed slight cell growth inhibition at 20 μM in PC-3 cells. Among the three hydrazone derivatives, none possessed very good antiproliferative activity in the cancer cell lines tested except compound **3**, which showed slight cytotoxic activity in LNCaP and PC-3 cells (IC_{50} of 20 and 25 μM, respectively). The effects of quinoline derivative **1** and its copper

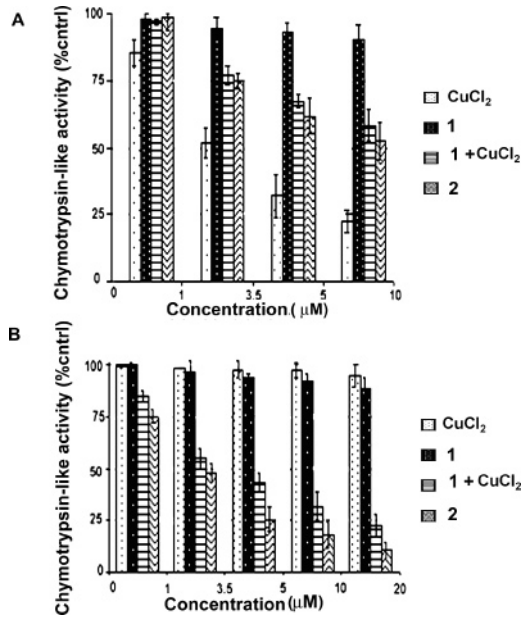


Figure 2. Inorganic copper complex mediated proteasome inhibition in vitro: (A) 20S purified proteasome; (B) LNCaP whole-cell extract after treatment with $CuCl_2$ alone, **1**, solution mixture of **1** with $CuCl_2$, and **2**.

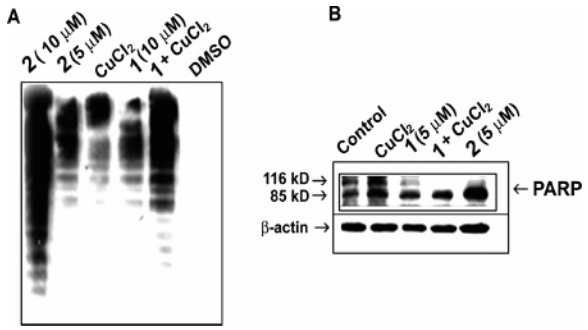


Figure 3. Inhibition of proteosome activity and apoptosis inhibition in prostate cancer LNCaP cells treated with DMSO for control, $CuCl_2$ alone, **1** (10 μM), solution mixtures of **1** and $CuCl_2$ (10 μM), and **2** (5 and 10 μM). (A) Western blot analysis for accumulation of ubiquitinated proteins as an indicator for proteosome inhibition. Treatment of LNCaP cells with (1) a solution mixture of **1** with $CuCl_2$ and (2) copper complex **2** alone results in ubiquitinated protein accumulation, suggesting proteosome inhibition. (B) Western blot analysis for cleavage of PARP as an indication of apoptosis. Treatment with **1** alone, solution mixture of **1** and $CuCl_2$, and copper complex **2** results in cleavage of PARP, indicating that these complexes are capable of inducing apoptosis.

complex **2** were evaluated by ELISA for the detection of apoptotic cell death in LNCaP cells after 72 h of treatment.

The IC_{50} concentrations of quinoline were correlated with the inhibition of cell growth because measured derivatives showed increased apoptosis (Figure 1C) by the MTT assay in all the cell lines tested. Our observations suggest that apoptosis ELISA assay could be useful for drug development and chemosensitivity assessment because it can distinguish clinically useful anticancer compounds from toxic compounds with a very potent IC_{50} (as in the case of **3**), and thus, it is a sensitive assay for detecting apoptosis induced by novel antitumor compounds. From these observations we concluded that the thiol side chain substitution at the C_2 position of the quinoline moiety upon copper complexation leads to generation of a highly potent antiproliferative compound. Previously, it had been reported that the thioester adduct, lactathione, leads to generation of an active form within Jurkat cells, which leads to proteasome inactivation.[32] It has been shown by Kikuchi and others that inhibition of proteasomal chymotrypsin-like activity is associated with induction of apoptosis in tumor cells.[33]

To investigate the effects of these novel quinoline derivatives **1**, **3**, **5**, and **7** and their copper complexes, we also carried out in vitro assays using 20S purified proteasome and intact LNCaP cells to assess the effects of the Schiff base copper complexes with $CuCl_2$ as control. Our results using 20S proteasome confirmed previous findings by Dou et al.[15] where $CuCl_2$ salt alone inhibited proteasome activity at an IC_{50} of 3.5 μM compared to the copper complex **2** and its ligand **1**, which showed an IC_{50} higher than 10 μM in inhibiting 20S proteasome (Figure 2A). However, when we tested the effects of the copper complex **2** and its ligand **1** with copper salt alone, our results in intact LNCaP cells revealed that the copper complex **2** and mixture of **1** and copper (IC_{50} of 3.2 and 4 μM, respectively) are more potent in inhibiting proteasome−chymotrypsin-like activity compared to **1** and $CuCl_2$ alone ($IC_{50} \geq 10$ μM), as shown in Figure 2B.

These observations show that the presence of a cytotoxic pharmacophore at position C_2 in the parent quinoline creates a far more potent anticancer agent. When these ligands are further complexed with metals such as copper, it leads to stable proteasome inhibitory molecules, as indicated by the cyclic voltammetric studies and the scan rate dependence of the Cu^{2+}/Cu^{1+} couple rather than physical mixtures of known quinones and copper.[16] Ligand **1** shows an irreversible peak at −1.00 V (Figure 1A in Supporting Information) and a peak due to the aroylhydrazone side chain around −0.55 to −0.75 V, whereas its copper complex **2** has a reversible redox peak centered at +0.33 to +0.37 V (Figure 1B in Supporting Information), indicating scan rate dependence of the Cu^{2+}/Cu^{1+} couple (Figure 1C in Supporting Information).

Owing to the inhibitory effect on PC-3 and LNCaP cell proliferation, copper complex **2** and its ligand **1** were selected for further investigation to test the in vitro effects of these compounds along with the inorganic copper metal alone in LNCaP cells. When LNCaP cells were treated with DMSO (vehicle control), copper salt alone, no accumulation of ubiquitinated proteins was observed. However, when the solution mixture of ligand **1** and copper (1:1) was tested on LNCaP cells, after 24 h, a moderate level of ubiquitinated proteins was seen compared to the ligand **1** or copper salt alone. A significant accumulation of ubiquitinated proteins (Figure 3A) was observed with the Schiff base copper complex **2** at 10 μM compared to a lower concentration (5 μM) of **2**, DMSO control, and the mixture of ligand **1** and copper (1:1). The accumulation of ubiquitinated protein was comparable between the Schiff base copper complex **2** and the physical mixture of **1** and $CuCl_2$, indicating that quinolines upon metal complexation yield potent molecules capable of inhibiting proteosome activity. Moreover, we observed that **2** shows the apoptosis-specific PARP cleavage, which was apparent within 24 h in LNCaP cells (Figure 3B). These results suggest that Schiff base copper compounds **2** can inhibit the proteasome and induce apoptosis in tumor cells.

Copper complexes have been reported to mediate cell death via oxidative stress through generation of hydrogen peroxide.[34,35] To determine whether the Schiff base quinoline derivative **1** and its copper complex **2** generated H_2O_2 in LNCaP cells during induction of apoptosis or acted via a different mechanism, we carried out an H_2O_2 assay using the Amplex Red hydrogen peroxide kit (Molecular Probes). The LNCaP cells were treated with three concentrations of **1** and **2** (4, 7, and 10 μM) for 24 h followed by measurement of H_2O_2 at various time points from 1 to 6 h. The absorbance values were normalized to control

and plotted as absorbance at 560 nm versus concentration in μM. The Schiff base quinoline derivative and its copper complex did not generate high levels of H_2O_2 (Figure 2A in Supporting Information). The standard H_2O_2 curve was plotted as shown in Figure 2B in Supporting Information. At 10 μM, copper derivative **2** shows an almost 2-fold lowered absorbance than 10 μM H_2O_2 as indicated by the plot in Figure 2A in Supporting Information. Thus, induction of apoptosis by these compounds in LNCaP cells is not through oxidative stress but through inhibition of the proteasome−ubiquitin pathway, resulting in the induction of apoptosis in LNCaP prostate cancer cells as shown by our results.

Conclusions

Nitrogen heterocyclic compounds have been used widely in the pharmaceutical industry, medicine, and agriculture for their biological activity because of their antimicrobial, antipyretic, anti-inflammatory, and anticancer properties. We have described the synthesis and structural characterization of novel copper quinoline-2-carboxaldehyde complexes. These synthesized Schiff base compounds are different with respect to their various functional groups attached to the quinoline ligand. The cytotoxic activity is also affected by the nature of the side chains at position C_2, and our in vitro findings show that these compounds have high antiproliferative activity against prostate cancer cell lines PC-3 and LNCaP. Furthermore, these compounds are capable of inducing apoptosis in prostate cancer cells without oxidative stress, as indicated by the H_2O_2 assay. The highest cytotoxic activity was observed for the copper complex **2** that inhibited chymotrypsin-like proteasome activity in intact prostate LNCaP cancer cells. Our study strongly suggests that the strategies adopted in modifying the parent ligand with introduction of cytotoxic thiocarbonyl side chains enhance the antitumor property with subsequent lowering of IC_{50} values. Furthermore, the Schiff base copper complex **2** can inhibit the proteasome and induce apoptosis as shown by PARP cleavage in prostate tumor LNCaP cells. In conclusion, the present work indicates that introduction of a thiocarbonyl group at the C_2 position in the quinoline moiety upon copper complexation leads to generation of a potent anticancer agent that can be used for targeting the ubiquitin−proteasome pathway for the treatment of prostate cancer.

Supporting Information Available: Experimental details for the determination of biological activity, spectral data, and elemental analysis results for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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