SUPPLEMENTARY MATERIAL

Cell apoptosis induced by ciprofloxacin based Cu(II) complexes: Cytotoxicity, SOD mimic and antibacterial studies

Bhupesh S. Bhatt*[1], Divyang H. Gandhi[2], Foram U. Vaidya[3], Chandramani Pathak[3] and Tushar N. Patel[2]

[1]*Department of Chemistry, Sardar Patel University,*
Vallabh Vidyanagar - 388 120, Gujarat (INDIA)
Corresponding address: bhupeshbhatt31@gmail.com, bs_bhatt@spuvvn.edu

[2]*C. U. Shah University, Wadhwancity-363035, Gujarat (INDIA)*

[3]*Department of Cell Biology, School of Biological Sciences and Biotechnology,*
Indian Institute of Advanced Research, Koba Institutional Area,
Gandhinagar-382007, Gujarat (INDIA)

Supplementary data 1. Material and Method

All chemicals of molecular biology grade were purchased commercially. RPMI-1640, Dulbecco's Phosphate Buffer Saline (DPBS), Fetal bovine serum (FBS), Penicillin- Streptomycin-Neomycin (PSN) antibiotic mixture, Annexin V-FITC/Propidium Iodide (PI) apoptosis detection kit were purchased from Life technologies, Invitrogen (USA). Cell culture grade dimethyl sulfoxide (DMSO, ≥99.7%), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue were purchased from Sigma-Aldrich (USA). Lactate-dehydrogenase (LDH) cytotoxicity detection kit was purchased from Takara (Clontech, Japan). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

Isatin (97%), *p*-fluoroaniline (99%), *p*-chloroaniline (98%), *m*-nitroaniline (97%), *o*-chloroaniline (>98%), *p*-bromoaniline (97%), *p*-anisidine (99%), *o*-anisidine (99%) and *p*-toluidine (>99%) were procured from Sigma Aldrich (India). Phenazine methosulphate (PMS),

NADH, nitroblue tetrazolium chloride (NBT) and Luria Broth were procured from Himedia (India).

IR spectra were obtained on a FT-IR Shimadzu instrument in the range 4000–400 cm^{-1} and sample was prepared as KBr pellets. The conductance measurement were carried out using conductivity meter model number E-660A. The electronic spectra were obtained on a UV-160A, Shimadzu (Japan) UV-visible spectrophotometer. The Gouy's balance was used for magnetic moment measurement. The metal content was estimated by spectrophotometric titration, in which sample solutions were prepared by decomposing organic matter of complex with acid mixture and making it to a total volume of 20 mL with double distilled water. Ten different sets of solutions were prepared by taking a fixed amount of complex solution (2 mL), 2 mL acetate buffer solution and varying aliquots of 0.0005 M EDTA and making it to a total volume of 10 mL with double distilled water. Absorbance was measured at 745 nm using buffer as a reference. Amount of copper was determine using the plot of absorbance against volume of EDTA.

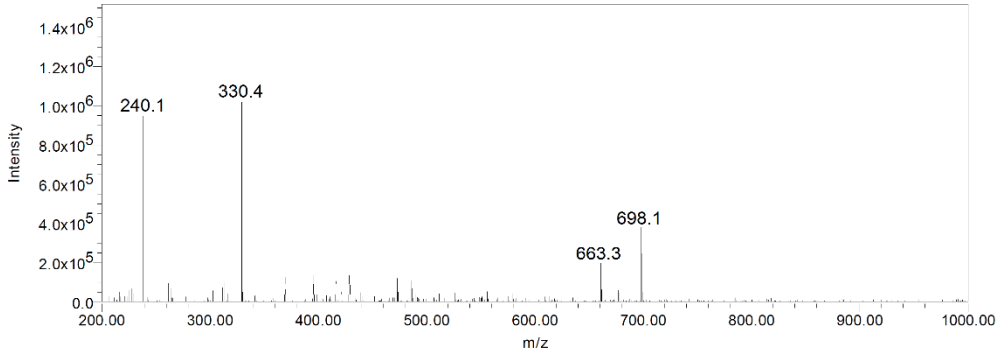
Supplementary data 2. Physical characterization data of complexes.

Complexes	ν(C=O) pyridone /cm^{-1}	$\nu(COO)_{as}$ /cm^{-1}	$\nu(COO)_{s}$ /cm^{-1}	Δν /cm^{-1}	ν(M-N) /cm^{-1}	ν(M-O) /cm^{-1}
Ciprofloxacin	1708	1624	1340	284	-	-
1	1618	1560	1353	207	542	518
2	1615	1567	1367	200	545	513
3	1629	1555	1358	197	540	504
4	1620	1567	1366	201	549	519
5	1612	1560	1362	198	535	512
6	1622	1565	1366	199	542	518
7	1630	1564	1358	206	540	515
8	1615	1555	1359	196	548	510

Supplementary data 3. IR spectra data of ciprofloxacin and compounds 1 - 8.

Complexes	Empirical formula	Formula weight	%Cu		m.p. /^{0}C	μ_{eff} /B.M	Molar conductivity/ $\mu S\ cm^{-1}$
			Theoritical	Experimental			
[Cu(CFL)(A^1)Cl] (1)	$C_{33}H_{32}ClCuF_2N_5O_4$	699.63	9.08	8.65	>250	1.85	20.8
[Cu(CFL)(A^2)Cl] (2)	$C_{33}H_{32}Cl_2CuFN_5O_4$	716.09	8.87	8.50	>250	1.87	21.9
[Cu(CFL)(A^3)Cl] (3)	$C_{33}H_{32}BrClCuFN_5O_4$	760.54	8.36	8.03	>250	1.83	23.1
[Cu(CFL)(A^4)Cl] (4)	$C_{34}H_{32}ClCuFN_5O_5$	711.67	8.93	8.68	>250	1.92	21.8
[Cu(CFL)(A^5)Cl] (5)	$C_{33}H_{32}ClCuFN_6O_6$	726.64	8.75	8.49	>250	1.85	22.0
[Cu(CFL)(A^6)Cl] (6)	$C_{34}H_{35}ClCuFN_5O_4$	695.67	9.13	8.80	>250	1.86	23.6
[Cu(CFL)(A^7)Cl] (7)	$C_{33}H_{32}Cl_2CuFN_5O_4$	716.09	8.87	8.56	>250	1.85	19.5
[Cu(CFL)(A^8)Cl] (8)	$C_{34}H_{35}ClCuFN_5O_5$	711.67	8.93	8.70	>250	1.98	21.1

Supplementary data 4. Mass spectra of compound – 1.



Supplementary data 5. Experimental

Cell lines and cell culture

Human colorectal carcinoma (HCT 116) cell line was obtained from National Center for Cell Science (NCCS), Pune, Maharashtra, India. HCT 116 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% PSN, an antibiotic mixture (Life technologies, USA). The cells were cultured in a humidified condition of 5% CO_2 at 37°C. The exponentially growing cells were used in entire study.

Treatments of Compounds

Compounds were freshly prepared in cell culture grade DMSO. Exponentially growing HCT 116 were treated with Compound 1 to 8 for 24 h. Cells were treated with DMSO considered as vehicle control.

Evaluation of half maximal inhibitory concentration

The half maximal inhibitory concentration (IC_{50}) of Compound 1 to 8 was determined by MTT assay as describe an earlier (Waghela, Sharma, Dhumale, Pandey & Pathak 2015). Briefly, 1 X 10^4 HCT-116 cells were treated with series of synthesized compounds (1 to 8) in different concentrations (1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL and 150 µg/mL) for 24h. Thereafter, the cells were washed with DPBS and incubated with MTT (5 mg/ml) for 1 h in dark at 37˚C. After incubation the MTT was removed and DMSO was added to each well. The absorbance was recorded at 570 nm with the reference wavelength of 650 nm by using Multimode microplate reader (SpectraMax M2e, Molecular Devices, USA). The results were represented as inhibition in cell proliferation and IC_{50} value was evaluated.

Morphological analysis

The morphological changes in HCT 116 cells after the treatments were observed by bright field microscopy. HCT 116 cells were treated with selective compound 2 (12 µg/mL and 24 µg/mL) for 24 h. More than 150 cells from three different fields were observed under bright field microscope (DP-71, IX81, Olympus, Japan). The magnification used for morphological analysis is 40X magnification under DIC (Differential interference contrast) filter of bright field microscope.

Cell viability assay

Cell death was evaluated by trypan blue exclusion assay as describe an earlier (Ranjan, Sharma, Surolia & Pathak 2014). Briefly, HCT 116 cells were treated with selective compound 2 (12 µg/mL and 24 µg/mL) for 24 h. The treated cells were harvested and washed with DPBS. Thereafter 10 µl of cell suspension was mixed with 10 µl of trypan blue dye. The dead cells and total cells were counted. The percentage of cell death was determined by the following formula (Percentage of cell death = Number of dead cells/Total number of cells x 100).

Colony formation assay

Colony formation assay is a cell survival assay, which shows the ability of a single cell to form a colony *in vitro* condition. The assay was performed by using crystal violet as describe an earlier (Waghela et al. 2015). HCT 116 cells were treated with selective compound 2 (12 µg/mL and 24 µg/mL) for 24 h. After completion of incubation cells were harvested and 1000 cells were seeded in 6 well plate. Cells were incubated for 10-12 days until the visible colonies were observed. Thereafter, colonies were fixed with methanol and stained with 0.2% crystal violet. After washing the excessive stain, plates were scanned and the colonies were counted. The percentage of Plating Efficiency was counted using the formula: Percentage plating efficiency (PE) = (Number of colonies formed/Number of cells seeded) x100.

Apoptosis assay

The apoptosis assay was performed by using Annexin V/PI staining. 1 x 10^5 HCT-116 cells were cultured on coverslip coated with Poly-L-Lysin in 24 well plate. Thereafter the cells were treated with Compound 2 (12 µg/mL and 24 µg/mL) for 24 h. After completion of incubation, cells were stained with 5 µL Annexin-V FITC for 20 mins and 1 µl of Propidium Iodide (PI) for 5 mins at room temperature in the dark. Thereafter, the cells were observed under a fluorescent microscope and more than 100 cells were taken from three random fields to examine the apoptotic cell death. All the images were acquired by Image-Pro MC 6.1, (Bethesda, MD, USA) and analyzed by Image J software (NIH, USA).

Cytotoxicity assay

The release of Lactate dehydrogenase (LDH) is known to consider the marker of necrotic cell death. Therefore, Lactate dehydrogenase (LDH) was quantified to distinguish between apoptotic and necrotic cell death as describe an earlier (Dhumale, Waghela & Pathak 2015). HCT 116 cells were also treated with 2mM H2O2 for 6 h which is used as a positive control. Briefly, HCT 116 cells were treated with Compound 2 (12 µg/mL and 24 µg/mL) and vehicle control (DMSO) for 24 h. The release of LDH was quantified by using a LDH assay kit (Takara, Shiga, Japan) as per the manufacturer's instructions.

***In vitro* antibacterial assay**

The *in vitro* bacteriostatic activity of the metal salt, ciprofloxacin, gatifloxacin and pefloxacin (standard drugs) and complexes against two Gram$^{(-ve)}$ *Escherichia coli, Serratia marcescens,* and two Gram$^{(+ve)}$ *Bacillus subtilis*, *Staphylococcus aureus*, microorganisms were evaluated in terms of minimum inhibitory concentration (MIC) using double dilution technique as reported procedure (Patel, Bhatt & Dosi 2012). The bacterial culture were incubated in Luria Broth as media at 37^{o} C, and bacterial growth was monitored by measuring the turbidity in solution after 18 h, in presence and absence of metal salt, standard and samples. The lowest concentration of compounds at which bacterial growth inhibited, was considered as the MIC value.

SOD-like activity

The assay is based on a fact that nitroblue tetrazolium (NBT) can be photoreduced to produce $O_2^{\bullet-}$, and the ability of complexes to scavenge $O_2^{\bullet-}$ at their vacant site can be the indicator of their SOD-mimic activity. The complex (0.25 to 5.0 µM), NADH (79 µM), NBT (75 µM), and PMS (30 µM) solution in phosphate buffer (pH 7.8) were used in this assay. The generated superoxide anion by this system were spectrophotometrically detected at 560 nm by monoformazan formation. The reduction rate of NBT was estimated in the presence and absence of the complexes after 5 min. The concentration of complexes responsible for 50% inhibition of NBT reduction was considered as IC_{50} value of complexes (Patel et al. 2012).

Brine shrimp lethality assay

Artemia Cysts eggs were hatched in artificial seawater for two days. A sample stock solution was preared by dissolving 10 mg complex in 10 mL DMSO. From this, solution were transferred to 18

different vials to make 2, 4, 8, 12, 16 and 20 µg mL^{-1} final concentrations of complexes, three vials were kept with DMSO as control only. The 10 nauplii in 1 mL of seawater were added and the volume was adjusted to 2.5 mL per vial with seawater. After 24 h, the survivor numbers were counted. The log of sample concentrations were plotted against %mortality of nauplii and LC_{50} value was determined (Islam et al. 2007).

References

Dhumale, S. S., Waghela, B. N. & Pathak, C. (2015). Quercetin protects necrotic insult and promotes apoptosis by attenuating the expression of RAGE and its ligand HMGB1 in human breast adenocarcinoma cells. *IUBMB life 67*: 361-373.

Islam, M. R., Islam, S. R., Noman, A. S. M., Khanam, J. A., Ali, S. M. M., Alam, S. & Lee, M. W. (2007). Biological screening of a novel nickel (II) tyrosine complex. *Mycobiology 35*: 25-29.

Patel, M. N., Bhatt, B. S. & Dosi, P. A. (2012). Spectroscopic study of DNA hydrolysis, DNA intercalative, and electrostatic interaction activity exerted by drug based coordination compounds. *Zeitschrift für anorganische und allgemeine Chemie 638*: 152-162.

Ranjan, K., Sharma, A., Surolia, A. & Pathak, C. (2014). Regulation of HA14-1 mediated oxidative stress, toxic response, and autophagy by curcumin to enhance apoptotic activity in human embryonic kidney cells. *BioFactors 40*: 157-169.

Waghela, B. N., Sharma, A., Dhumale, S., Pandey, S. M. & Pathak, C. (2015). Curcumin conjugated with PLGA potentiates sustainability, anti-proliferative activity and apoptosis in human colon carcinoma cells. *PloS one 10*.