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# Chiral anionic binuclear zinc complexes based on diaminocyclohexane ligand and their in vitro antiproliferative studies



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# ABSTRACT

Two novel binuclear chiral anionic Zn(II) complexes,  $[Zn_2LCl_3]^- (C_2H_5)_3N^+ 1$  and  $[Zn_2L(CH_3COO)_3]^- (C_2H_5)_3N^+ 2$  counterbalanced by triethyl ammonium cation, have been synthesized from ligand,  $2,2'-((1E,1'E)-((1R,2R)-cyclohexane-1,2-diylbis(azanylylidene))bis(methanylylidene))dipheno, H_2L. The ligand, H_2L and its complexes are characterized by elemental analyses, IR, <sup>1</sup>H and <sup>13</sup>C NMR, ESI-MS, electronic and thermal studies. Complex$ **1** $has been additionally characterized by single crystal X-ray diffraction studies and confirmed a slightly distorted tetragonal pyramid coordination environment in which both zinc atoms are five coordinated by two imine nitrogen atoms, two oxygen atoms and one chloride ion located at the polyhedron apex. Ligand, H_2L and its Zn(II) complexes have been subjected to antiproliferative studies against HCT p53 wild type as well as HCT p53 null cell lines, and the results suggest complexes to be an effective antiproliferative agent against these cancer cell lines in comparison to ligand, H_2L. Furthermore, DNA fragmentation studies revealed that Zn(II) complexes induce significant p53 independent apoptosis in cancer cell lines.$ 

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# 1. Introduction

The clinical success of cisplatin and related platinum based anticancer drugs encouraged the researchers to discover more selective and less toxic metallopharmaceuticals [1,2]. The important problems associated with the use of cisplatin in curative therapy are the severe toxic side effects viz., nephrotoxicity, neurotoxicity, ototoxicity, low water solubility, instinct, and acquired resistance manifested in various types of cancers [1–3]. To date, a large number of metal-based chemotherapeutic agents have been developed to treat or cure a variety of cancers but most of them demonstrate restricted efficacy due to the problem of delivery and penetration, and low selectivity for the tumor cells causing severe damage to healthy tissues compared with other transition metals [1,2]. Therefore, researchers have diverted their attention towards the synthesis of molecularly-targeted rationally designed metal-based drugs to minimize unwanted side effects as well as to improve tumor selectivity while retaining the desirable therapeutic effectiveness [4]. Among transition metals, zinc is the most prominent trace metal in human body after iron and plays an important role in various biological processes, and act as a major regulatory ion in the metabolism of cells in human body [1–3,5,6]. A search of literature reveals that zinc is cytoprotective and suppresses apoptotic pathways, and has shown a significant efficacy in the prevention of colon and thyroid cancer through induction of cell cycle arrest and apoptosis [1,2,7–10]. In recent years, zinc-containing compounds are regarded as one of the most promising alternatives to cisplatin as anticancer drugs. Magda et al. have synthesized several water-soluble complexes of zinc ionophore 1-hydroxypyridine-2-thione (ZnHPt), and propose them to be a potential new class of anticancer agents [11]. In order to widen the scope of investigations on new biologically active pharmaceuticals of zinc, we hereby design the synthesis of some novel chiral binuclear anionic zinc complexes derived from chiral salen ligand, obtained by the condensation of o-hydroxybenzaldehyde and (1S,2S)-(+)-1,2diaminocyclohexane. Literature reveals that chiral salen-type ligands and their complexes have been studied extensively because of their wide applications in various areas of research viz., medical sciences, non-linear optical materials, asymmetric catalysis and magnetic materials [7,12]. The synthesized and isolated salen ligand, H<sub>2</sub>L and its Zn(II) complexes have been characterized by elemental analyses and various spectroscopic studies viz., UV-Vis, IR, NMR, ESI-MS spectrometry and thermal investigation. Complex 1 has been additionally characterized by single crystal X-ray diffraction measurement. Moreover, anti-proliferative effects of the synthesized compounds have been studied on HCT p53 wild type as well as HCT p53 null cell lines. The results revealed that the compounds induce significant apoptosis in p53 independent manner in cancer cells, which is further confirmed by DNA fragmentation.

# 2. Experimental

The starting materials, (1S,2S)-(+)-1,2-diaminocyclohexane, zinc chloride, zinc acetate and o-hydroxybenzaldehyde were purchased from Aldrich. All other reagents and solvents were of high purity and used as purchased without any further purification.

# 2.1. Physical measurements

C, H, and N elemental analyses were recorded on Elementar Varrio EL analyzer. FT-IR ( $4000-400 \text{ cm}^{-1}$ ) spectra were obtained as a KBr

pellet using Perkin Elmer 621 spectrophotometer. <sup>1</sup>H and <sup>13</sup>C NMR spectra of ligand and its Zn(II) complexes were recorded in CDCl<sub>3</sub> using JEOL 400 spectrometer. Mass spectrometry was performed using Agilent technologies ion trap LC/MS 6320 mass spectrometer with electrospray positive ionization mode. Electronic spectra of the complexes were obtained in methanol on Pharmacia LKB-Biochem, UV/Vis spectrophotometer at room temperature. Thermal behavior of the synthesized compounds was studied by using SDTQ-600 (TA Instrument) in helium atmosphere (100 mL min<sup>-1</sup>) at heating rate of 20 °C/min at temperature 20–800 °C.

#### 2.2. Synthesis of salen ligand: H<sub>2</sub>L

The salen ligand was prepared according the methods reported in the literature [13]. A methanolic solution of 2-hydroxybenzaldehyde (2 mmol) was added dropwise to the methanolic solution of (1S,2S)-(+)-1,2-diaminocyclohexane (1 mmol). The reaction mixture was stirred for 5 h resulting into a yellow colored solution, which was concentrated to 1 ml followed by the addition of 20 ml of diethyl ether to cause precipitation. The precipitate was removed by filtration, and the filtrate was evaporated to get analytically pure compound.

Yield 78%, Color: Yellow, Mp. 120 °C; Molecular formula  $C_{20}H_{22}N_2O_2$ ; Anal Calc. C, 74.50; H, 6.88; N, 8.69% Found: C, 74.45; H, 6.82; N, 8.65% <sup>1</sup>HNMR (CDCl<sub>3</sub>):  $\delta$  (ppm) 13.32 (Ar–OH), 8.25 (s – CH=N), 6.78–7.24 (m Ar–H), 3.29 (H(a) N–CH–CH–N), 3.32 (H(a) N–CH–CH–N), 195–1.88 (m, – CH<sub>2</sub>–CHN–CHN–CH<sub>2</sub>–), 1.87–1.46 (m, –CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CHN–CHN–CH<sub>2</sub>);  $\delta$  (ppm) 161.0 (–CH=N), 164.7 (–C–OH), 132.2–116.8 (Ar–C), 72.6 (N–CH–CH–N), 33.1 (–CH<sub>2</sub>–CHN–CHN–CH<sub>2</sub>–), 24.2 (–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–), ESI-MS (M + Na)<sup>+</sup> (m/z), 323.2, IR, 2990 cm<sup>-1</sup>  $\nu$ <sub>(Ar–OH)</sub>, 1690 cm<sup>-1</sup>  $\nu$ <sub>(CH=N)</sub>, 1185  $\nu$ <sub>(Arc–O)</sub>.

# 2.3. Synthesis of complex, **1** $[Zn_2LCl_3]^- (C_2H_5)_3 N^+$

A solution of zinc chloride (2 mmol) in methanol was added dropwise into 10 ml methanol solution of ligand (1 mmol). The resultant reaction mixture was stirred for 5 h followed by addition of triethylamine (1 mmol) with constant stirring. The resulting solution was refluxed for 2 h and then left for evaporation at room temperature. After few days, yellow colored crystals suitable for single crystal X-ray diffraction were separated out.

Yield 75%, Color: Yellow, Mp. 215 °C; Molecular formula  $C_{26}H_{36}N_3O_2$ Cl<sub>3</sub>Zn<sub>2</sub>; Anal Calc. C, 47.33; H, 5.49; N, 6.37% Found: C, 47.31; H, 5.45; N, 6.33%, <sup>1</sup>HNMR (CDCl<sub>3</sub>):  $\delta$  (ppm) 8.45 (s – CH=N), 6.79–7.25 (m Ar–H), 3.42 (H(a) N–CH–CH–N), 3.43 (H(a) N–CH–CH–N), 197–1.92 (m, –CH<sub>2</sub>–CHN–CHN–CH<sub>2</sub>–), 1.90–1.49 (m, –CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–C, <sup>13</sup>CNMR (CDCl<sub>3</sub>):  $\delta$  (ppm) 164.6 (–CH=N), 167.5 (–C–OH), 142.8–121.3 (Ar–C), 74.4 (N–CH–CH–N), 35.3 (–CH<sub>2</sub>–CHN–CHN–CH<sub>2</sub>–), 25.1 (–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–CH<sub>2</sub>–), ESI-MS (M + Na)<sup>+</sup> (m/z), 659.7, IR, 1620 cm<sup>-1</sup>  $\upsilon$ <sub>(CH=N)</sub>, 1195  $\upsilon$ <sub>(ArC–O)</sub>, 570 cm<sup>-1</sup>  $\upsilon$ <sub>(Zn–N)</sub>.

## 2.4. Synthesis of complex, **2** $[Zn_2L(CH_3COO)_3]^- (C_2H_5)_3N^+$

A methanol solution of  $Zn(OAc)_2 \cdot 2H_2O$  (2 mmol) was added dropwise into 10 ml methanol solution of ligand (1 mmol). The resultant reaction mixture was stirred for 5 h followed by addition of triethylamine (1 mmol) with constant stirring. The resulting solution was heated under refluxing conditions for 2 h and then evaporated at room temperature leading to the formation of yellow colored microcrystalline solid product. No crystal was found suitable for single crystal X-ray diffraction.

Yield 71%, Color: Yellow, Mp. 198 °C; Molecular formula  $C_{32}H_{44}N_3O_8Zn_2$ ; Anal Calc. C, 52.69; H, 6.07; N, 5.76% Found: C, 52.65; H, 6.03; N, 5.72%, <sup>1</sup>HNMR (CDCl<sub>3</sub>):  $\delta$  (ppm) 8.35 (s -CH=N), 6.47-7.54 (m Ar-H), 3.68 (H(a) N-CH-CH-N), 3.67 (H(a') N-CH-CH-N), 1.94-1.91 (m, -CH<sub>2</sub>-CHN-CHN-CH<sub>2</sub>-), 1.88-1.86 (m, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-), <sup>13</sup>CNMR (CDCl<sub>3</sub>):  $\delta$  (ppm) 161.0 (-CH=N), 164.7 (-C-OH), 116.8-132.2 (Ar-C), 72.6 (N-CH-CH-N), 58.4 (-CH<sub>2</sub>-CHN-CHN-CH<sub>2</sub>-), 56.4 (-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-), 56.4 (-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-), 56.4 (n-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>

# 2.5. X-ray determination of complex 1

The vellow prism crystal of complex **1** was sealed in glass capillary filled with helium and next it was mounted on a KM-4-CCD automatic diffractometer equipped with CCD detector, and used for data collection. X-ray intensity data were collected with graphite monochromated  $CuK_{\alpha}$  ( $\lambda = 1.54178$  Å) radiation at temperature 291.0(3) K, with  $\omega$  scan mode. The exposure time used was 33 s, and reflections inside Ewald sphere were collected up to  $\theta = 72.29^{\circ}$ . The unit cell parameters were determined from 1360 strongest reflections. Details concerning crystal data and refinement are given in Table 1. Examination of reflections on two reference frames monitored after each 20 frames measured showed no loss of the intensity during measurement. Lorentz, polarization and numerical absorption [14] corrections were applied during the data reduction. The structure was solved by partial structure expansion procedure. All the non-hydrogen atoms were refined anisotropically using full-matrix, least-squares technique on  $F^2$ . All the hydrogen atoms were found from difference Fourier synthesis after four cycles of anisotropic refinement, and refined as "riding" on the adjacent atom with geometric idealization after each cycle of refinement and individual isotropic displacement factors equal to 1.2 times the value of equivalent displacement factor of the parent non-methyl carbon and nitrogen atoms, and 1.5 times of parent methyl carbon atoms. The methyl groups were allowed to rotate about the local three-fold axes. The Flack parameter was refined as full matrix parameter. The SHELXS97, SHELXL97 and SHELXTL [15] programs were used for all the calculations.

#### Table 1

Crystal and structure refinement data of complex 1.

Compound	1
Empirical formula	$C_{26}H_{36}Cl_3N_3O_2Zn_2$
Formula weight	659.67
Crystal system, space group	Monoclinic, P2 <sub>1</sub> (No. 4)
Unit cell dimensions [Å, °]	a = 15.6822(3)
	b = 9.7400(2)
	c = 19.5376(3)
	$\beta = 105.372(1)$
Volume [Å <sup>3</sup> ]	2877.50(9)
Z, calculated density [Mg/m <sup>3</sup> ]	4, 1.523
Absorption coefficient [mm <sup>-1</sup> ]	4.836
F(000)	1360
Crystal size [mm]	0.099, 0.083, 0.082
$\theta$ range for data collection [°]	2.35 to 72.29
Index ranges	$-19 \le h \le 19, -10 \le k \le 11,$
	$-24 \le 1 \le 24$
Reflections collected/unique	$30,930/9718 [R_{(int)} = 0.0325]$
Completeness [%]	99.8 (to $\theta = 67^{\circ}$ )
Min. and max. transmission	0.647 and 0.675
Data/restraints/parameters	9718/1 <sup>a</sup> /655
Goodness-of-fit on F <sup>2</sup>	1.123
Final <i>R</i> indices $[I > 2\sigma(I)]$	R1 = 0.0243, $wR2 = 0.0628$
R indices (all data)	R1 = 0.0244, $wR2 = 0.0629$
Largest diff. peak and hole $[e \cdot Å^{-3}]$	0.280, -0.998

<sup>a</sup> Floating origin restrain

Table 2			
Colocted structural data of compound	1	r	Å

|--|--|

Zn1-02	2.0408(15)	Zn31-N31	2.0393(19)
Zn1 – N1	2.0534(17)	Zn31-031	2.0399(15)
Zn1-01	2.0547(17)	Zn31-032	2.0446(17)
Zn1-N2	2.0759(19)	Zn31 – N32	2.0831(17)
Zn1-Cl1	2.2484(6)	Zn31 – Cl31	2.2474(6)
Zn1-Zn2	3.0896(4)	Zn31 – Zn32	3.0805(4)
Zn2-02	1.9796(18)	Zn32-031	1.9793(17)
Zn2-01	1.9893(14)	Zn32-032	1.9801(14)
Zn2-Cl2	2.2067(6)	Zn32 – Cl32	2.2029(5)
Zn2-Cl3	2.2501(6)	Zn32 – Cl33	2.2395(6)
N1-C7	1.276(3)	C37 – N31	1.283(3)
N2-C14	1.281(3)	N32-C44	1.277(3)
02-Zn1-N1	142.47(7)	N31-Zn31-O31	87.49(7)
02-Zn1-01	77.52(6)	N31-Zn31-O32	143.62(7)
N1-Zn1-01	87.17(7)	031-Zn31-032	77.55(6)
02 - Zn1 - N2	86.65(7)	N31-Zn31-N32	80.20(7)
N1-Zn1-N2	80.29(7)	O31-Zn31-N32	131.70(7)
01 – Zn1 – N2	134.91(7)	O32-Zn31-N32	85.38(7)
02 – Zn1 – Cl1	110.77(5)	N31-Zn31-Cl31	106.67(5)
N1 – Zn1 – Cl1	106.75(5)	O31-Zn31-Cl31	117.40(5)
01 – Zn1 – Cl1	113.73(5)	O32-Zn31-Cl31	109.66(5)
N2 – Zn1 – Cl1	111.36(6)	N32-Zn31-Cl31	110.87(5)
02 - Zn2 - 01	80.49(7)	O31-Zn32-O32	80.49(6)
02 - Zn2 - Cl2	115.30(5)	O31-Zn32-Cl32	115.45(5)
01 - Zn2 - Cl2	118.61(5)	O32-Zn32-Cl32	114.12(5)
02 - Zn2 - Cl3	110.00(5)	031 - Zn32 - Cl33	112.22(5)
01 – Zn2 – Cl3	112.29(5)	032 - Zn32 - Cl33	112.85(5)
Cl2 – Zn2 – Cl3	115.24(2)	Cl32 – Zn32 – Cl33	116.54(2)

Atomic scattering factors were those incorporated in the computer programs. Selected interatomic bond distances and angles are listed in Table 2 and intermolecular interactions are listed in Table 3.

#### 2.6. Method for determination of in vitro antiproliferative activity

#### 2.6.1. Cell culture

HCT 116 (human colon carcinoma) cell lines having p53 wild type (+/+) and p53 null (-/-) status were cultured in DMEM medium supplemented with 10% FBS and maintained at 37 °C under 5% CO<sub>2</sub> atmosphere and 95% humidity. Both cell lines were subcultured every third day or after reaching 70% confluency.

#### 2.6.2. MTT assay

Cells intended for MTT assay were trypsinized and counted on improved Neubaur's Chamber after staining with Trypan Blue to quantify viable cells. Around 5000 viable cells were plated per well in a 96 well plate and incubated for 24 h followed by their treatment with varying concentration of ligand, H<sub>2</sub>L and complexes **1** and **2** for 48 h. Henceforth, MTT assay kit of Invitrogen Bioservices was used to perform MTT assay of treated cells. DMSO was used as control.

#### 2.6.3. IC<sub>50</sub> determination

Mean absorbance values from MTT assay were used to determine half maximal inhibitory concentration ( $IC_{50}$ ) values for each synthesized compounds after 48 h of treatment. The first step, percentage

Table 3	
Hydrogen bond geometry of compound <b>1</b> [Å, °].	

$D - H \cdot \cdot A$	d(D-H)	d(H…A)	d(D…A)	<(DHA)
N61-H61N-Cl3	0.97	2.23	3.1479(19)	158.7
N71-H71N-Cl33	0.94	2.23	3.1644(18)	172.1
C14-H14-Cl32	0.93	2.74	3.457(2)	135.0
C65-H65B-Cl31	0.97	2.79	3.676(3)	152.8
C75 – H75A – Cl1 <sup>i</sup>	0.97	2.72	3.653(3)	160.9

Symmetry transformations used to generate equivalent atoms: (i) x + 1, y, z.

cell viability after drug treatment at each drug concentration was determined using the formula-

Percentage Cell viability =  $\frac{\text{Mean absorbance of treated samples}}{\text{Mean absorbance of untreated samples}} \times 100\%.$ 

Next, regression analysis tool in Microsoft Excel software was used to determine regression for the line plotted between drug concentration (x-axis) and percentage cell viability (y-axis) and consequently values for intercept (c) and slope (m). Finally, value of x (half maximal inhibitory concentration for the drug) was calculated from the formula y = mx + c after putting the value of y = 50 (for 50% inhibition of cell viability following drug treatment) with other values (c, m) known from previous step.

# 2.6.4. DNA fragmentation assay

After reaching about 50% cell confluency in a 100 mm tissue culture plate, culture medium of the cells was replaced with fresh culture medium containing either synthesized compounds, ligand, H<sub>2</sub>L and complexes **1** and **2** (at 100  $\mu$ M), DMSO, cisplatin (at 50  $\mu$ M) or simply fresh culture medium in case of untreated sample and incubated for 48 h to allow drug treatment. Each sample was then processed for DNA extraction using DMSO (dimethyl sulphoxide)–SDS (sodium dodecyl sulfate)–TE (Tris–EDTA) method described elsewhere [16]. The amount of DNA and its purity in each sample obtained after extraction were determined using UV–spectroscopy by recording absorbance at 260 and 280 nm. DNA samples along with gel loading dye were loaded onto 2% agarose gel to a final DNA concentration of 10  $\mu$ g and run in 0.5 × TAE buffer at 95 V. Cisplatin (50  $\mu$ M) treated sample was used as a positive control. 1 kb DNA ladder was used in marker lane.

# 3. Results and discussion

The free salen ligand,  $H_2L$  has been prepared by the condensation of (1S,2S)-(+)-1,2-diaminocyclohexane and 2-hydroxybenzaldehyde in 1:2 molar ration in methanol [Scheme 1]. Literature reveals that salen ligands have strong chelating ability with various metal ions [17]. Most significantly, salen complexes themselves can act as ligands for further coordination with another metal substrate [18]. Usually, the oxygen atoms serve as donor atoms and, further coordinate to second metal ion [19]. The corresponding binuclear anionic Zn(II) complexes,

counterbalanced by triethylammonium cation, were synthesized by the reaction of ligand,  $H_2L$  with Zn(II) ions in 1:2 molar stoichiometry in methanol [Scheme 1].

#### 3.1. Spectroscopic studies

The IR spectrum of ligand, H<sub>2</sub>L shows a broad band at 2990 cm<sup>-1</sup> attributed to  $v_{(\Omega-H)}$  of phenol. The band due to  $v_{(Ar-O)}$  observed at  $1160 \text{ cm}^{-1}$  in the free ligand is shifted to higher frequency and appears at 1190 cm<sup>-1</sup> and 1195 cm<sup>-1</sup> for complexes **1** and **2**, respectively, suggesting its coordination to Zn(II) ion [Fig. 1S] [20,21]. A strong band at 1650 cm<sup>-1</sup> ascribed to  $v_{(C=N)}$  in the free ligand suffers negative shift upon coordination and appears at 1620  $\text{cm}^{-1}$  for complex **1** and 1640  $\text{cm}^{-1}$  for complex **2**, respectively, indicating the coordination of azomethine nitrogen to Zn(II) ion [21,22]. The complex **2** shows stretching bands for the acetate ion at 1595 cm<sup>-1</sup> and 1390 cm<sup>-1</sup>, corresponding to the asymmetric and symmetric vibration of the carboxylate moiety, respectively. The frequency difference of approximately 200 cm<sup>-1</sup> between asymmetric and symmetric vibration suggests a monodentate coordination mode for the acetate group [21,23]. The electronic spectra of the ligand and its Zn(II) complexes exhibited absorption bands at 255 nm and 310 nm, assigned to  $\pi$ - $\pi$ \* and n- $\pi$ \* transitions, respectively. Furthermore, absorption bands observed at 270 nm and 345 nm for complex 1 and, 260 nm and 350 nm for complex 2, respectively, may be assigned to metal charge transfers for their d<sup>10</sup> electronic configuration. The bands at lower wave length correspond to intraligand charge transfers, whereas other bands originate from ligand to metal charge transfers [Fig. 2S] [24]. The <sup>1</sup>HNMR spectrum of ligand, H<sub>2</sub>L revealed a sharp resonance signal from azomethine hydrogens at 8.25 ppm (s 2H) and a singlet at 13.32 ppm (s 1H) due to OH resonances. Resonances from phenyl hydrogens were observed at 6.78–7.24 ppm. Signals from cyclohexane bridge protons N-CH-CH-N were recorded as multiplets at 3.29 ppm (1H) H(a), and 3.32 ppm (1H) H(a') due to diastereotopic coupling in the cyclohexane ring, while the chemical shifts for the cyclohexane ring protons appeared as multiplets at 1.46-1.95 ppm [Fig. 3S]. Upon coordination of free ligand, H<sub>2</sub>L to Zn(II) ion, the hydroxyl signal disappeared indicating deprotonation of the hydroxyl groups due to Zn - O bond formation. Resonance signals from cyclohexane bridge CH protons were shifted towards higher frequencies and appeared at 3.33 ppm H(a) and 3.30 ppmH(a') for complex 1 and, 3.68 (1H) (Ha), and 3.67 (1H) H(a') for complex 2, respectively [Fig. 4S]. The <sup>13</sup>CNMR spectrum of free ligand





 $H_2L$ 

Scheme 1. Zn(II) complexes prepared in this study; complex 1 with  $ZnCl_2 \cdot 2H_2O$  and complex 2 with  $Zn(OAc)_2 \cdot 2H_2O$ .

X = CI. OAc



Fig. 1. The molecular structure of complex 1 plotted with 50% probability of displacement ellipsoids. The hydrogen atoms were omitted for clarity.

revealed azomethine signal at 164.6 ppm, whereas resonance signals for cyclohexane bridge carbon appeared at 72.5 ppm for C(a) and at 76.6 for C(a'), respectively. Signals from aromatic carbons and cyclohexane carbons were registered at 116.6–132.3 ppm and 24.0–33.0 ppm, respectively [Fig. 5S]. These values undergo significant shift upon coordination to Zn(II) ion [Fig. 6S]. The structure of the ligand, H<sub>2</sub>L and its Zn(II) complexes were also supported by mass spectrometry under positive ion ESI condition. The molecular ion peaks for the ligand and its Zn(II) complexes were in agreement with their assigned molecular formulae. The ESI-MS spectrum of the synthesized ligand showed the presence of the molecular ion peak as a sodium adduct  $[M + Na]^+$ , m/z at 323.2, while its corresponding calculated m/z being 322.4 [Fig. 7S]. The ESI-MS spectra of complexes **1** and **2** showed molecular ion peak at 659.7, 729.4, respectively.

#### 3.2. Thermogravimetric analyses

Thermal stability of complexes **1** and **2** were carried out by thermogravimetric analyses (TGA and DTG) in helium atmosphere at heating rate of 20 °C min<sup>-1</sup> within the temperature 20–800 °C. These compounds undergo three distinct steps of weight loss [Fig. 8S]. The TGA of complex **2** shows first weight loss of 15% corresponding to moisture and acetate ion at 20–220 °C. Further elevation within temperature 220–445 °C leads to rapid weight loss ca. 53% attributed to the dissociation of the salen ligand at the imine bond. A gradual weight loss at temperature 445–800 °C leads to the complete decomposition of organic moiety and leaves a final residue of ZnO. Thus the total weight loss up to 800 °C is found to be 85%. The TGA data is further supported by DTG, which shows three main steps at 20–220 °C, 200–445 °C and 445–800 °C. The maximum weight loss occurred in the second stage at 220–445 °C. The result of TGA and DTG clearly indicated that the second stage is the main degradation stage.

# 3.3. X-ray structural description of complex 1

A perspective view of complex **1** structure is shown in Fig. 1. The compound is composed of complex anion  $[Zn_2Cl_3(C_{20}H_{20}N_2O_2)]^-$ , and charge balancing triethylammonium cation. All atoms occupy the general positions. The asymmetric part of the unit cell contains the two ion pairs (two cations and two anions) of **1**, and these pairs are related by non-crystallographic pseudosymmetry center. In general, only three pairs of carbon atoms (C8:C43, C10:C41, C13:C38) are not interrelated by symmetry center, due to chiral centers located at four of them. The other parts of the anions (including heavy atoms) and whole cations are mutually symmetrically deepened. This affects the reflection statistics, which rather shows the centrosymmetric behavior than the non-centrosymmetric one. Additionally the cations and part of anions (with exclusion of cyclohexane rings) possess the internal pseudosymmetry planes going through the Zn1/Zn2/Cl1/Cl2/Cl3, Zn31/Zn32/ Cl31/Cl32/Cl33, N61/C63/C64 and N71/C73/C74 atoms. The all chiral centers (located at C8, C13, C38, and C43 atoms) have S configuration. The both anions and both cations possess almost identical conformations, and the root mean square deviations of superimposed ions are 0.090(2) and 0.102(2) Å, respectively (the most distant equivalent atoms C9:C39 and C64:C74 are separated at 0.218(2) and 0.182(2) Å, respectively). The Zn1 and Zn31 atoms are five-coordinated by two imine nitrogen atoms, two alkoxide oxygen atoms and one chloride anion, and the coordination environment of the metal atoms can be described as slightly distorted tetragonal pyramid [25] with the chloride anion located at the polyhedron apex. The Zn1 and Zn31 atoms exist in the distorted tetrahedral [25] coordination environment created by two alkoxide anions and two chloride anions. Thus each chloride anion is terminal-monofunctional and each alkoxide anion is bridging. The 2-hydroxidebenzylideneamino moieties of both molecules of 1 are close to planarity, and however some obvious deviations of atoms from the respect weighted least square planes exist, and they are not larger than 0.10 Å, except one atom (C44) which deviates 0.124(2) Å

Table 4	
Table depicting percentage cell	viability after drug treatment

1 61 6 9	8					
Drug compound $\Longrightarrow$	F1		F2		FOAc	
p53 status of cell>	++		++		++	
Drug concentration (in µM)						
↓						
35	110.7802%	79.9275%	-	-	-	-
50	99.60464%	76.4259%	107.0638%	66.87411%	88.16552%	62.7974%
75	90.0%	71.8056%	98.86663%	53.35986%	85.01845%	55.0772%
100	-	-	75.90933%	47.7706%	74.0485%	44.8046%
IC50 values (in $\mu$ M) $\Longrightarrow$	152.4596	182.8271	145.53	90.708	189.7939	86.7435



Fig. 2. Treatment on HCT p53 wild type cell line. Graph plotted between  $H_2L$  concentrations on x-axis and mean absorbance at 570 nm (depicting percentage of viable cells after treatment) on y axis.

from respect plane. Within each molecule above mentioned planes are inclined at 20.27(6) and 21.75(6)°, respectively for molecules containing N1 and N31 atoms. The cyclohexane rings show almost ideal boat conformation, with the C<sub>s</sub> asymmetry parameters [25] (placed on opposite ring atoms) in range 0.60–4.05, and  $C_2$  asymmetry parameters [25] (placed on opposite ring bonds) in range 1.11–5.40. The analysis of C – N bond lengths shows that the double bonds are fully localized within benzylideneamino moieties (Table 2). Each cation is bonded to one complex anion via N-H-Cl intermolecular hydrogen bond (Table 3). The ions of complex 1 are additionally connected by C-H-Cl interactions, which can be classified as weak hydrogen bonds [26,27] (Table 3). Altogether these interactions form the supramolecular hydrogen-bonded chain extending along crystallographic [001] axis. The stacking interactions are not observed within the crystal structure of 1 (the aromatic rings are separated by aliphatic moieties of cation and anions).

# 3.4. In vitro antiproliferative assay

# 3.4.1. Discussion for MTT

Anti-proliferative effects of ligand,  $H_2L$  and complexes **1** and **2**, as compared to DMSO control, were studied on HCT p53 wild-type as well as HCT p53 null cell lines. 24 h of treatment by either of the compounds was unable to cause a significant effect on proliferative potential



# HCT +/+ Complex 1 48 h

Fig. 3. Complex 1 treatment on HCT p53 wild type cell line for 48 h. Graph plotted between complex 1 concentration on x-axis and mean absorbance at 570 nm (depicting percentage of viable cells after treatment) on y-axis.



**Fig. 4.** Complex **2** treatment on HCT p53 wild type cell line. Graph plotted between complex **2** concentration on x-axis and mean absorbance at 570 nm (depicting percentage of viable cells after treatment) on y-axis.

of both HCT p53 wild type as well as HCT p53 null cell lines. However after 48 h of treatment, complexes **1** (at 100  $\mu$ M) and **2** (at 50, 75 as well as 100  $\mu$ M) exhibited a clear anti-proliferative effect on HCT p53 wild type cell line, although the drug concentration required for a significant effect on the cell line by both these drugs still remained high as reflected by their IC<sub>50</sub> values of 145.53  $\mu$ M and 189.79  $\mu$ M for complexes **1** and **2**, respectively (Table 4). Inhibitory effect of these compounds was much more pronounced in HCT p53 null cell line after 48 h of treatments at all the three doses used for each compound, when compared to DMSO control. At 100  $\mu$ M concentration, complexes **1** and **2** exhibited inhibition up to about 50% as compared to DMSO control, while H<sub>2</sub>L showed inhibition up to 25% at 75  $\mu$ M. This increase in inhibitory capability of complexes **1** and **2** was also obvious by a significant decrease in their IC<sub>50</sub> value, as compared to that in case of p53 wild type cell line,



Fig. 5.  $H_2L$  treatment on HCT p53 null cell line. Graph plotted between  $H_2L$  concentration on x-axis and mean absorbance at 570 nm (depicting percentage of viable cells after treatment) on y-axis.



**Fig. 6.** Complex **1** treatment on HCT p53 null cell line. Graph plotted between complex **1** concentration on x-axis and mean absorbance at 570 nm (depicting percentage of viable cells after treatment) on y-axis.



**Fig. 7.** Complex **2** treatment on HCT p53 null cell line. Graph plotted between complex **2** concentration on x-axis and mean absorbance at 570 nm (depicting percentage of viable cells after treatment) on y-axis.

with values dropping to 90.71  $\mu$ M and 86.74  $\mu$ M for complexes **1** and **2** respectively. H<sub>2</sub>L was not as effective as either complex **1** or **2** in inhibiting cancer cell growth, as evident by its high IC<sub>50</sub> values of 152.46  $\mu$ M and 182.83  $\mu$ M in case of p53 wild type and p53 null cancer cell lines respectively. A lower concentration range for ligand, H<sub>2</sub>L had to be taken because of its lower solubility than complexes **1** and **2** and therefore to reach 100  $\mu$ M concentration, more volume of compound solution (dissolved in DMSO) had to be used [Figs. 2–7]. Now for DMSO (used to solubilize compounds here), more than 0.1% (v/v) concentration is not advised. Hence, it appears that p53 null cell line is more sensitive to ligand H<sub>2</sub>L and complexes **1** and **2** as compared to p53 wild type cell line, detailed mechanism for which needs to be investigated.

# 3.5. Discussion for DNA fragmentation assay

After studying anti-proliferative activity of compounds with MTT assay, we proceeded to show whether the synthesized compounds induce apoptosis in p53 wild type and p53 null status cells. The nucleosome excision or DNA fragmentation is induced much earlier than changes in cell morphology as cell progresses through apoptosis [28]. Among various methods to detect DNA fragmentation, DNA ladder assay (agarose gel electrophoresis) is the most frequently used technique used for apoptosis detection, since DNA fragment ladder obtained is hallmark of apoptotic cells [29,30]. In present assay, we ran negative control as untreated cells and positive control as cisplatin treated cells along with compounds and DMSO treated cells. Lane loaded with cisplatin treated cell's DNA shows faint bands of fragmented DNA as expected. Low number of cells may result in such faint band lanes of



HCT (+/+) cells

**Fig. 8.** For HCT (+/+) cells: Lane 1–marker (1 kb), Lane 2–untreated cells (negative control), Lane 3–DMSO treated cells, Lane 4–H<sub>2</sub>L treated cells, Lane 5–complex **1** treated cells, Lane 6–complex **2** treated cells, Lane 7–cisplatin treated cells (positive control).



**Fig. 9.** For HCT (-/-) cells: Lane 1-marker (1 kb), Lane 2-untreated cells (negative control), Lane 3-DMSO treated cells, Lane 4-H<sub>2</sub>L treated cells, Lane 5-complex 1 treated cells, Lane 6-complex 2 treated cells, Lane 7-cisplatin treated cells (positive control).

cells treated with synthesized compounds that show faint bands at same positions as cisplain treated lane and thus suggests that DNA fragmentation (apoptosis) is induced in drug treated samples too [Figs. 8, 9]. Laddering is more prominent in case of HCT p53 null status cell line which correlates with what is suggested by MTT assay earlier.

#### 4. Conclusion

In this paper, we attempted to design the synthesis and structural characterization of two novel chiral anionic binuclear Zn(II) complexes, counterbalanced by triethylammonium cation. The ligand, 2,2'-((1E,1'E)-((1R,2R)-cyclohexane-1,2-diylbis(azanylylidene))bis(methanylylidene)) diphenol is reasonably easy to prepare and its complexation with Zn(II) ions produce complexes with slightly distorted tetragonal pyramidal geometry in which both Zn atoms are five coordinated by the two imine nitrogen atom, two oxygen atom and one chloride ion located at the polyhedron apex. The uncoordinated ligand as well as its corresponding Zn(II) complexes have been tested in vitro towards cancer cell lines: HCT p53 wild type and HCT p53 null cell lines. Results suggest ligand to be less active, while Zn(II) complexes show promising anticancer activity against these cancer cell lines. Furthermore, studies on DNA fragmentation revealed that the compounds induce significant p53 independent apoptosis in cancer cell lines. Finally preliminary investigations implicated in apoptosis and growth arrest indicate a key role played by these Zn(II) complexes in the control of cell proliferation and/or cell arrest. Therefore, we can conclude that the above mentioned Zn(II) complexes may be candidates for the further assessment as a chemotherapeutic agent for human cancers.

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# Appendix A. Supplementary material

**CCDC905280** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk./ data\_request/cif. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.inoche. 2014.05.029.

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