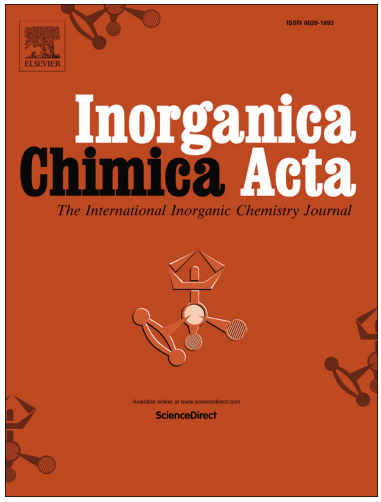

Research paper

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Novel N-Heterocyclic Carbene Silver(I) Complexes: Synthesis, Structural Characterization, and Anticancer Activity

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Abstract

In this study, we synthesized four novel unsymmetrically substituted NHC ligands (**1a-d**) and their Ag(I) complexes (**2a-d**). All new compounds were characterized using elemental analysis, FT-IR, ^{1}H NMR, and ^{13}C NMR spectroscopy and X-ray crystallography. The molecular structure of complex **2d** was elucidated through single crystal X-ray diffraction analyses. Single crystal structural studies for complex **2d** show that the benzene rings (C9-C14) and the central benzimidazole ring system make dihedral angles of 85.65(11)°. The Ag-C*l* and Ag-C single bond lengths are 2.3553(7) and 2.096(2) Å, respectively. The C-Ag-C*l* bond angle is 168.27(7)°. . Both salts and complexes were tested for their anti-cancer potential against three human cancer cell lines (DU-145, MCF-7, and MDA-MB-231) and non-cancer cells adipose from mouse (L-929) for 24h, 48h and 72 h using the MTT assays. However, the Ag(I)-NHC complexes **(2a-d)** showed a dose and time-dependent cytotoxic activity against all cell lines. MDA-MB-231 human breast carcinoma cells were the most sensitive to the Ag(I)-NHC complex displaying IC_{50} lower than 1 μM all time points. Further, the IC_{50}s for Ag(I)-NHC were higher in non-cancer cells, suggesting that complexes possessed noteworthy selectivity for human cancer cells.

Keywords: N-Heterocyclic carbene; silver complex; benzimidazole-2-ylidene; anticancer activity.

1. INTRODUCTION

Cancer is a major public health problem worldwide and is the second leading cause of death. According to GLOBOCAN 2012, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008 [1]. One of the primary approaches to cancer treatment is chemotherapy. Unfortunately, chemotherapy is not always effective and is associated with

severe side effects [2]. Cisplatin was on of the first metal-based anticancer drugs, also called metallotherapeutic drugs. Cisplatin spearheaded thousands of studies seeking to identify novel metal-based anticancer therapeutics. A primary goal of these studies is the identification of compounds with increased efficacy, but reduced side effect.

Heterocyclic azoles, such as imidazole, triazole, and especially benzimidazole have significant biological activities [3-7]. Especially, *N*-heterocyclic carbenes (NHC) derived from the imidazole or benzimidazole core which have considerable antibacterial or anticancer activities [8-13]. NHCs are also a well-known class of organometallic ligands which are a strong σ-donating and weak π-accepting when connected with metal ions. Metal-NHC complexes have exhibited an interesting propensity to bind DNA by means of non- covalent interactions, which is believed to partially mediate their anti- proliferative activity, and to cleave DNA strands [14-17]. In addition to their antimicrobial and anticancer activity, metal-NHC complexes have also been intensely investigated due to their noteworthy applications in catalysis [18-21].

Silver has lower toxicity as compared to other transition metals. One reason for this could be the slower the rate of release of silver ions, from the metallodrug complexes, which also enhanced their biological activity. Among the many developed anticancer agents consisting of organometallic compounds, those based on silver–NHC complexes have attracted much attention because of their biological properties such as antimicrobial, anticancer, and antibacterial activities [13, 22-27]. Ag-NHC complexes have been suggested that as antimicrobial agents to prevent infections, in wound care antiseptics and in water purification [28,29]. Metallic silver was used as a treatment for gonorrhea before the development of antibiotics, while silver nitrate was commonly used as an antimicrobial compound before the discovery of penicillin in ancient times. [30]. Silver nitrate is still being used as a preventive drug against the development of neonatal conjunctivitis in infants.

With the above in mind, we report the synthesis and characterization of four new benzimidazolium salts and their Ag(I) complexes. All the new synthesized compounds (**1a-d, 2a-d**) were tested as potential anticancer agents against three human cancer cell lines (DU-145, MCF-7, MDA-MB-231) and non-cancer cells adipose from mouse (L-929) for 24 h, 48 h and 72 h by MTT assay [31].

2. EXPERIMENTAL

2.1. Materials and Methods

All manipulations were carried out under argon in flame-dried glassware using standard Schlenk line techniques. The silver (I) oxide Ag_2O, benzimidazole, and allyl bromide were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The solvents were dried by conventional methods and were distilled immediately prior to use and were transferred under Argon [32]. Elemental analyses were performed by Inönü University Scientific and Technology Center (Malatya/Turkey). Microlab. Melting points determined using Electrothermal 9100 melting point detection apparatus. Fourier transform infrared (FTIR) spectra were obtained in the range 400–4000 cm^{-1} on Perkin Elmer Spectrum 100 FT-IR. ^1H NMR and ^{13}C NMR spectra were recorded using a Varian As 400 Mercury spectrometer operating at 400 MHz (^1H), 100 MHz (^{13}C) in $CDCl_3$. with tetramethylsilane as the internal reference. ^1H peaks are labeled as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Chemical shifts and coupling constants are reported in ppm and in Hz, respectively. The intensity data were collected using a Bruker Kappa ApexII CCD diffractometer using Mo-K_α radiation (λ= 0.71073 Å) [33]. The structure was solved by direct methods using the solution program SHELXS97, and then refined with full-matrix least-square methods based on F^2 *SHELXL2014*/6 [34, 35]. All the measurements were taken at room temperature for freshly prepared solutions

The cells lines DU-145 (HTB-81, human prostate carcinoma), MCF-7 (HTB-22, human breast adenocarcinoma), MDA-MB-231 (HTB-26, human breast adenocarcinoma), F-12K Medium (30-2004), Eagles Minimum Essential Medium (EMEM, 30-2003), RPMI-1640 (30-2001), fetal bovine serum (FBS, 30-2020) and penicillin and streptomycin (30-2300) were purchased from American Type Culture Collection (ATTC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM, D6429) and Trypsin-EDTA solution (T-3924) were purchased from Sigma Aldrich. L-929 (non-cancer cells adipose from mouse) were purchased from ECACC (European Collection of Animal Cell Culture, Salisbury, U.K.). All absorbance values were measured with a microplate reader (Epoch, USA) at 570 nm.

2.2 General procedure for the benzimidazolium salts, 1a-d

Benzimidazole (10 mmol) was added to a solution of NaH (10 mmol) in dry THF (30 mL), the mixture was stirred for 1 h at room temperature. Allyl bromide (10.1 mmol) was added slowly to the solution and heated for 24 h at 60 ^0C. All solvent was evaporated under the vacuum. After that, dichloromethane (50 mL) was added to solid and the solution was

filtered. The obtained clear solution was distilled. 1-Allyl benzimidazole was obtained. The 1-allyl benzimidazole (1 mmol) and alkyl halide (1 mmol) were stirred in DMF (5 mL) for 24 h at 80 ^{0}C. White product was precipitated. The solution was filtered, solid was rinsed out with diethylether and dried under vacuum. The crude product was recrystallized from dichloromethane/diethylether [36].

1-(Allyl)-3-(2-methylbenzyl)benzimidazolium chloride, 1a

Yield: 92%; mp 176-177 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.35 (s, 3H, $CH_2C_6H_4(CH_3)$-2), 5.30-5.31 (m, 2H, NCH_2CHCH_2), 5.39-5.43 (m, 2H, NCH_2CHCH_2) (5.84 (s, 2H, $CH_2C_6H_4(CH_3)$-2), 6.02-6.12 (m, 1H, NCH_2CHCH_2), 7.05-7.20 (m, 4H, $CH_2C_6H_4(CH_3)$-2), 7.36 (d, 1H, NC_6H_4N, J = 8 Hz), 7.44 (t, 1H, NC_6H_4N, J = 6 Hz), 7.52 (t, 1H, NC_6H_4N, J = 6 Hz), 7.66 (d, 1H, NC_6H_4N, J = 8 Hz), 11.80 (s, 1H, NCHN). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 19.6 ($CH_2C_6H_4(CH_3)$-2), 50.0 ($CH_2C_6H_4(CH_3)$-2), 50.2 (NCH_2CHCH_2), 113.7 (NCH_2CHCH_2), 121.7 (NCH_2CHCH_2), 113.9, 126.8, 127.1, 127.2, 128.2, 129.3, 129.7, 131.3 and 136.5 ($CH_2C_6H_4(CH_3)$-2 and NC_6H_4N), 144.3 (NCHN); $\nu_{(CN)}$: 1553 cm^{-1}; % Anal. Calcd for $C_{18}H_{19}N_2Cl$: C, 72.35; H, 6.41; N: 9.38; Found: C, 72.33; H, 6.39; N, 9.37.

1-(Allyl)-3-(3-methylbenzyl)benzimidazolium chloride, 1b

Yield: 91%; mp 133-134 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.32 (s, 3H, $CH_2C_6H_4(CH_3)$-3), 5.36 (d, 2H, NCH_2CHCH_2, J = 8 Hz), 5.51-5.54 (m, 2H, NCH_2CHCH_2), 5.96 (s, 2H, $CH_2C_6H_4(CH_3)$-3), 6.09-6.19 (m, 1H, NCH_2CHCH_2), 7.14 (d, 1H, $CH_2C_6H_4(CH_3)$-3, J = 8 Hz), 7.23-7.30 (m, 3H, $CH_2C_6H_4(CH_3)$-3), 7.52-7.62 (m, 3H, NC_6H_4N), 7.72 (d, 1H, NC_6H_4N, J = 8 Hz), 11.68 (s, 1H, NCHN). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.3 ($CH_2C_6H_4(CH_3)$-3), 50.2 ($CH_2C_6H_4(CH_3)$-3), 51.6 (NCH_2CHCH_2), 113.7 (NCH_2CHCH_2), 121.7 (NCH_2CHCH_2), 113.9, 125.3, 127.0, 127.1, 128.9, 129.2, 129.8, 130.0, 131.4, 132.7 and 139.3 ($CH_2C_6H_4(CH_3)$-3 and NC_6H_4N), 143.6 (NCHN); $\nu_{(CN)}$: 1561 cm^{-1}; % Anal. Calcd for $C_{18}H_{19}N_2Cl$: C, 72.35; H, 6.41; N: 9.38; Found: C, 72.32; H, 6.39; N, 9.36.

1-(Allyl)-3-(4-methylbenzyl)benzimidazolium chloride, 1c

Yield: 89%; mp 119-120 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.30 (s, 3H, $CH_2C_6H_4(CH_3)$-4), 5.34 (d, 2H, NCH_2CHCH_2, J = 4 Hz), 5.44-5.50 (m, 2H, NCH_2CHCH_2), 5.86 (s, 2H, $CH_2C_6H_4(CH_3)$-4), 6.07-6.17 (m, 1H, NCH_2CHCH_2), 7.15 (d, 2H, $CH_2C_6H_4(CH_3)$-4, J = 8 Hz), 7.42 (d, 2H, $CH_2C_6H_4(CH_3)$-4, J = 8 Hz), 7.52-7.60 (m, 2H,

NC_6H_4N), 7.66 (d, 1H, NC_6H_4N, J = 8 Hz), 7.73 (d, 1H, NC_6H_4N, J = 8 Hz), 11.79 (s, 1H, NC*H*N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.2 ($CH_2C_6H_4(CH_3)$-4), 50.1 ($CH_2C_6H_4(CH_3)$-4), 51.3 (NCH_2CHCH_2), 113.7 (NCH_2CHCH_2), 121.7 (NCH_2CHCH_2), 113.9, 127.1, 128.4, 129.7, 129.8, 130.0, 131.5 and 139.1 ($CH_2C_6H_4(CH_3)$-4 and NC_6H_4N), 143.5 (NCHN); $\nu_{(CN)}$: 1558 cm^{-1}; % Anal. Calcd for $C_{18}H_{19}N_2Cl$: C, 72.35; H, 6.41; N: 9.38; Found: C, 72.33; H, 6.39; N, 9.36.

1-(Allyl)-3-(2-chlorobenzyl)benzimidazolium chloride, 1d

Yield: 89%; mp 122-123 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.38 (d, 2H, NCH_2CHCH_2, J = 4 Hz), 5.45-5.50 (m, 2H, NCH_2CHCH_2), 6.04 (s, 2H, $CH_2C_6H_4$Cl-2), 6.11-6.18 (m, 1H, NCH_2CHCH_2), 7.31-7.35, 7.41-7.44, 7.54-7.62 and 7.66-7.74 (m, 8H, $CH_2C_6H_4$Cl-2), 11.79 (s, 1H, NCHN). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 48.6 ($CH_2C_6H_4$Cl-2), 50.3 (NCH_2CHCH_2), 113.7 (NCH_2CHCH_2), 121.7 (NCH_2CHCH_2), 113.8, 127.2, 127.3, 128.1, 129.7, 130.1, 130.3, 130.9, 131.2, 131.3, 131.4 and 133.6 ($CH_2C_6H_4$Cl-2 and NC_6H_4N), 144.3 (NCHN); $\nu_{(CN)}$: 1558 cm^{-1}; % Anal. Calcd for $C_{17}H_{16}N_2Cl_2$: C, 63.96; H, 5.05; N: 8.78; Found: C, 63.98; H, 5.06; N, 8.80.

2.3 General procedure for preparation of Ag(I)-NHC complexes, 2a-d

A solution of benzimidazolium salt (0.5 mmol) (**1a–d**) and Ag_2O (1.0 mmol) in dichloromethane (15 mL) was stirred at room temperature for 24 h in the dark condition and the suspension was passed filtered through celite. The solvent removed under vacuum. The crude product was recrystallized from dichloromethane/diethyl ether.

Chloro[1-allyl-3-(2-methylbenzyl)benzimidazole-2-ylidene]silver(I), 2a

Yield: 86%; mp: 176-178 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.39 (s, 3H, $CH_2C_6H_4(CH_3)$-2), 5.10 (d, 2H, J = 4 Hz, NCH_2CHCH_2), 5.24 (d, 1H, J = 16 Hz, NCH_2CHCH_2), 5.34 (d, 1H, J = 8 Hz, NCH_2CHCH_2), 5.62 (s, 2H, $CH_2C_6H_4(CH_3)$-2), 5.98-6.10 (m, 1H, NCH_2CHCH_2), 6.74 (d, 1H, J = 8 Hz, NC_6H_4N), 7.06-7.13 (m, 1H, $CH_2C_6H_4(CH_3)$-2), 7.22-7.24 (m, 3H, $CH_2C_6H_4(CH_3)$-2, 7.36-7.40 and 7.28-7.32 (m, 2H, NC_6H_4N), 7.50 (d, 1H, J = 8 Hz, NC_6H_4N). ^{13}C{H}-NMR (100 MHz,$CDCl_3$) δ : 19.6 ($CH_2C_6H_4(CH_3)$-2), 51.4 (NCH_2CHCH_2), 52.1 ($CH_2C_6H_4(CH_3)$-2), 111.9 (NCH_2CHCH_2), 119.2 (NCH_2CHCH_2), 112.1, 124.3, 126.4, 126.6, 126.8, 128.4, 131.0, 131.8, 132.7, 133.8, 134.0 and 135.4 (NC_6H_4N and ($CH_2C_6H_4(CH_3)$-2), 189.9 ($C_{carbene}$-Ag); $\upsilon_{(CN)}$: 1387 cm $^{-1}$; Anal. Calcd for $C_{18}H_{18}AgClN_2$: C, 53.29; H, 4.47; N: 6.91. Found: C, 53.22; H, 4.78; N, 6.95; Found: C, 53.26; H, 4.44; N: 6.89; LC–MS: 631.1 $[AgL_2]^+$.

Chloro[1-allyl-3-(3-methylbenzyl)benzimidazole-2-ylidene]silver(I), 2b

Yield: 78%; mp 157-158 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.31 (s, 3H, $CH_2C_6H_4(CH_3)$-3), 5.07-5.10 (m, 2H, NCH_2CHCH_2), 5.24 (d, 1H, NCH_2CHCH_2, J = 16 Hz), 5.34 (d, 1H, NCH_2CHCH_2, J = 8 Hz), 5.58 (s, 2H, $CH_2C_6H_4(CH_3)$-3), 5.98-6.08 (m, 1H, NCH_2CHCH_2), 7.04-7.12 and 7.19-7.23 (m, 4H, $CH_2C_6H_4(CH_3)$-3),7.30-7.39 and 7.46-7.48 (m, 4H, NC_6H_4N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.4 ($CH_2C_6H_4(CH_3)$-3), 52.1 ($CH_2C_6H_4(CH_3)$-3), 53.5 (NCH_2CHCH_2), 112.0 (NCH_2CHCH_2), 119.3 (NCH_2CHCH_2), 112.2, 124.3, 124.4, 127.8, 129.0, 131.8, 133.8, 134.0 and 139.0 ($CH_2C_6H_4(CH_3)$-3 and NC_6H_4N), $C_{carbene}$-Ag: not observed; $\nu_{(CN)}$: 1391 cm^{-1}; % Anal. Calcd for $C_{18}H_{18}N_2AgCl$: C, 53.29; H, 4.47; N: 6.91; Found: C, 53.27; H, 4.43; N: 6.89; LC–MS: 631.1 $[AgL_2]^+$.

Chloro[1-allyl-3-(4-methylbenzyl)benzimidazole-2-ylidene]silver(I), 2c

Yield: 79; mp 153-154 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.31 (s, 3H, $CH_2C_6H_4(CH_3)$-4), 5.06-5.09 (m, 2H, NCH_2CHCH_2), 5.21-5.34 (m, 2H, NCH_2CHCH_2), 5.58 (s, 2H, $CH_2C_6H_4(CH_3)$-4), 5.98--6.05 (m, 1H, NCH_2CHCH_2), 7.12-7.24, 7.27-7.37 and 7.45-7.47 7.15 (m, 8H, $CH_2C_6H_4(CH_3)$-4 and NC_6H_4N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.1 ($CH_2C_6H_4(CH_3)$-4), 52.1 ($CH_2C_6H_4(CH_3)$-4), 53.4 (NCH_2CHCH_2), 111.9 (NCH_2CHCH_2), 119.3 (NCH_2CHCH_2), 112.2, 119.3, 124.3, 127.2, 129.8, 131.8, 133.8 and 138.4 ($CH_2C_6H_4(CH_3)$-4 and NC_6H_4N), 188.8 ($C_{carbene}$-Ag); $\nu_{(CN)}$: 1395 cm^{-1}; % Anal. Calcd for $C_{18}H_{18}N_2AgCl$: C, 53.29; H, 4.47; N: 6.91; Found: C, 53.26; H, 4.42; N: 6.88; LC–MS: 631.1 $[AgL_2]^+$.

Chloro[1-allyl-3-(2-chlorobenzyl)benzimidazole-2-ylidene]silver(I), 2d

Yield: 83%; mp 143-144 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.11-5.13 (m, 2H, NCH_2CHCH_2), 5.23-5.36 (m, 2H, NCH_2CHCH_2), 5.73 (s, 2H, $CH_2C_6H_4$Cl-2), 5.99-6.07 (m, 1H, NCH_2CHCH_2), 6.92-6.94, 7.15-71.9, 7.26-7.28, 7.34-7.42 and 7.46-7.51 (m, 8H, $CH_2C_6H_4$Cl-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 50.5 ($CH_2C_6H_4$Cl-2), 52.2 (NCH_2CHCH_2), 112.0 (NCH_2CHCH_2), 119.4 (NCH_2CHCH_2), 112.1, 124.6, 127.5, 128.3, 129.9, 130.1, 131.7, 132.3, 132.9 and 133.8 ($CH_2C_6H_4$Cl-2 and NC_6H_4N), 189.7 ($C_{carbene}$-Ag); $\nu_{(CN)}$: 1387 cm^{-1}; % Anal. Calcd for $C_{17}H_{15}N_2$ $AgCl_2$: C, 47.92; H, 3.55; N: 6.57; Found: C, 47.90; H, 3.57; N, 6.59; LC–MS: 671.1 $[AgL_2]^+$.

2.4. Cell viability (MTT) assay

DU-145 cells were grown in EMEM media containing 10% (v/v) FBS, and 100 Units/ml penicillin and 100 μg/ml streptomycin in a 37°C humidified incubator with 5% CO_2. MCF-7 cells, MDA-MB-231, and L-929 cells were grown under the same conditions except that DMEM and RPMI-1640 media were used, respectively. Cells were passaged at 70–80% confluence, about twice a week by trypsinization. The passages of all cells lines were no more than 30 during all experiments. The cytotoxic effect of newly synthesized ligands and complexes were determined using MTT [31]. A stock solution of the tested compounds was prepared in DMSO and diluted in complete culture medium such that the maximum DMSO content did not exceed 0.5% (v/v). Exponentially growing DU-145, MCF-7, MDA-MB-231, and L-929 cells were seeded into 96-well plates at a density of 1 x 10^5 cells/well and allowed to attach for 24h before treatment. Then the cells were treated with various concentration (1–20 μM) of compounds in 5% CO_2, at 37°C for 24h, 48 h, and 72 h. The control wells contained cells with media and 0.5% DMSO (final concentration of DMSO) and were kept the same in this study. At the end of the exposure period, the cells were subjected to assessment of viability by adopting the MTT assay. 10 μL/well MTT (5 mg/mL, dissolved in phosphate-buffered saline) was added and the cells were incubated for 2 h at 37°C in 5% CO_2. After removal of the medium and MTT, the purple-blue precipitated crystals were dissolved in 100 μL of DMSO (Sigma, St. Louis, MO). The absorbance was read at 570 nm with Biotek plate reader (Biotek. GA). Data were is based on the means from at least three independent experiments, each comprising three replicates per concentration. The anticancer effects of all compounds were determined in the concentration range of 1-20 μM, the dose-response curves were fitted by means of GraphPad Prism 7 (GraphPad Software, San Diego,CA, USA) and the IC_{50} values were calculated. Graphs indicating the cytotoxic effects of **1a-d,** and **2a-d** on DU-145, MCF-7, MDA-MB-231, and L-929 cells.

2.5. Statistical analysis

All experiments were carried out in triplicates and results are expressed as means ± SEM. Data were analyzed using one-way analysis of variance and differences were considered significant at $p < 0.0001$. The IC_{50} were determined by statistical software, GraphPad Prism7 (GraphPad Software, San Diego, CA, USA).

3. RESULTS AND DISCUSSIONS

3.1. Chemistry

Benzimidazolium salts were synthesized as shown in Scheme 1. Benzimidazolium salts were obtained from the reaction of allyl substituted benzimidazolium precursors with alkyl halides

in THF at 80 ^{0}C. Collapsed products were crystallized in dichloromethane/diethylether for purification.

<<Scheme 1 is here>>

Ag(I)-NHC complexes were synthesized from reaction benzimidazolium salts with Ag_2O in dichloromethane at room temperature under dark condition as shown in Scheme 2. Crude products were purified by crystallization in dichloromethane/diethylether [36]. The structures of all new compounds were characterized by elemental analysis using FT-IR, ^{1}H NMR, and ^{13}C NMR spectroscopy. The structure for compound **2d** was assessed using X-ray crystallography.

<<Scheme 2 is here>>

FT-IR spectra demonstrated, values in the range at 2872-2985 and 3011-3149 cm^{-1} belonging to the aliphatic and aromatic C-H stretching vibrational bands, respectively, for all compounds. C=C stretching vibration modes of all compounds were observed at around 1591-1667 cm^{-1}. Benzimidazole ring C=N vibrations of benzimidazolium salts (**1a-d**) showed peaks at around 1547-1559 cm^{-1}. Benzimidazole ring C=N vibrations of Ag(I)-NHC complexes (**2a-d**) were seen at around 1385-1396 cm^{-1}. After complexation, C=N vibrations of Ag(I)-NHC complexes shifted to the lesser energy region. This negative shift is because of the electropositive metal center which pulls electron density towards itself.

NMR spectra of all the compounds were analyzed in d-$CDCl_3$. In the ^{1}H NMR spectra, acidic proton (NCHN) for benzimidazolium salts (**1a-d**) were seen at 11.80, 11.68, 11.79 and 11.79 ppm, respectively, as a characteristic sharp singlet. Benzimidazolium salts lost their acidic proton when they formed silver complexes. This was shown by the disappearance of the acidic proton during ^{1}H NMR. Aromatic protons of benzimidazolium salts (**1a-b**) were detected in the range of 7.05-7.74 ppm. -C*H*- protons of allyl group on all benzimidazolium salts where in the range of 6.02-6.19 ppm and were observed as multiplets. -NCH_2 protons allyl group on the benzimidazolium salts (**1a-b**) appeared at 5.39-5.43 as mutiplet, 5.36, 5.34 and 5.38 as a singlet, respectively. Terminal -CH_2 protons of allyl group gave peaks in the range at 5.30-5.54 ppm as multiplets. Benzylic -CH_2 protons at benzimidazolium salts (**1a-d**) were observed at 5.84, 5.96, 5.86 and 6.04 ppm as a sharp singlet. Methyl protons of benzimidazolium salts (**1a-c)** signaled at 2.35, 2.32 and 2.30 ppm as singlets. The ^{13}C NMR spectra showed aromatic carbons of benzimidazolium salts (**1a-d**) in the range of 113.8-139.3 ppm. N*C*HN carbons on salts (**1a-d**) were observed at 144.3, 143.6, 143.5 and 144.3 ppm,

respectively. Terminal -CH_2 and –CH- carbons of the allyl group of all benzimidazolium salts gave peaks at 113. 7 and 121.7 ppm, respectively. For **1a-d** salts, -NCH_2 carbons on allyl groups appeared at 50.2, 51.6, 51.3 and 50.3 ppm, respectively. Observed peaks at 50.0, 50.2, 50.1 and 48.6 ppm were attributed to benzylic carbons of benzyl groups (**1a-b**), respectively. Methyl carbons on the benzyl group of salts **(1a-c)** were observed at 19.6, 21.3 and 21.2 ppm, respectively. ^{1}H NMR spectra and ^{13}C NMR spectra of benzimidazolium salts (**1a-d**) were compatible with each other.

It was observed that the ^{1}H NMR and ^{13}C NMR spectra of Ag(I)-NHC complexes detected peaks in the same regions of spectra corresponding to benzimidazolium salts, except for two characteristic peaks. The first of these corresponded to the disappearance of acidic proton peaks in the spectra of Ag(I)-NHC complexes, which confirmed that Ag(I)-NHC complexes (**2a-d**) were synthesized. The second of these, N*C*N carben resonance of the Ag(I)-NHC complexes (**2a-d**), which shifted to a downfield region as compared to the corresponding benzimidazolium salts (**1a-d**). Carben peaks of Ag(I)-NHC complexes **2a**, **2c** and **2d** were observed at 189.9, 188.8 and 189.7 ppm but were somewhat small, as these peak was not observed for **2b.** The lower values, or the absence of these peaks, are in agreement with reported data for similar Ag(I)-NHC complexes [37].

3.2. X-ray crystallography for 2d

The details of crystalization of complex **2d** are given below (Figure 1). All non-hydrogen atoms were refined isotropically first and then with anisotropic atomic displacement parameters by full matrix least square methods. All the hydrogen atoms bonded to carbon atoms were placed geometrically. All hydrogen atoms were refined as riding with U_{eq}(H)=1.2 U_{iso}(C). The Empirical Formula = $C_{17}H_{15}AgCl_2N_2$; Molecular Weight= 426.08 g.mol^{-1}; Crystal System = Triclinic; Space Group= *P*-1 system; UnitCell Parameters, *a*= 9.0290(7), *b*= 9.9785(9), *c*= 10.3038(9) Å; α = 94.964(3)^o, β = 100.682(3)°, γ = 114.349(3)^o; Cell Volume= 817.22(12) Å^3, *Z*= 2, *d* = 1.732 g.cm^{-3}, μ= 1.557 mm^{-1}. *F*(000)= 424; *T*= 296(2) K. The Crystal Size (0.43 × 0.38 × 0.36 mm). The number of measured reflections are 20104 with 3503 independent reflections. The observed [$I > 2\sigma(I)$] reflections are 3325. A final refinement on F^2, with 3503 unique intensities and 199 parameters. The value of weight parameter is *w*=0.063 and goodness of fit *S* = 1.09. Some selected parameters are given in Table 1. The revelant crystallographic for **2d** has been deposited at Cambridge Crystallographic Data Center (CCDC) with No. 1821504. The See the Supplementary

Materials for more details on the experimental methods, as well as complete NMR, FT-IR, LC-MS data and X-ray crystallography studies

<<Table 1. is here>>

<<Figure 1 is here>>

3.3. MTT Assays results

The anticancer potential of four newly synthesized benzimidazolium salts **(1a-d)** and their Ag(I)-NHC complexes **(2a-d)** were evaluated using the MTT assay on DU-145, MCF-7, and MDA-MB-231 cancer cells, as well as L-929 non-cancer cells. MTT staining assesses the ability of cells to convert a soluble yellow tetrazolium salt (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into insoluble purple formazan crystals, which is facilitated by mitochondrial dehydrogenase enzymes [31]. The effects of these benzimidazolium compounds on MTT were evaluated after 24, 48, and 72 h of exposure. Table 2 list the IC_{50} values of compounds for 48 h. (See the Supplementary Data, Table S1, for 24 h and 72 h IC_{50} values).

<<Table 2 is here>>

The IC_{50} values of all salts were > 20 µM at 24 h but decreased to 3.85 for some cells after to 48 h and to 1.86 after 72 h. MDA-MB-231 cells were the most susceptible to **1b** and **1c** salts which displayed IC_{50} values of 5.17 and 3.85 µM after 48 h respectively. Notably, the IC_{50} values of **1c** for 48 h and 72 h were lower than those of **1a, 1b,** and **1d**, suggesting higher antiproliferative activity than **1a, 1b** and **1d.** The silver complexes **(2a-d)** decreased MTT staining in all cells, as compared to controls. Similar to the salts, a decrease in MTT staining were concentration and time-dependent. The silver complexes **(2a-d)** showed better antiproliferation activity with IC_{50} (concentration of the test compound to achieve 50% inhibition) ranging from <1 to 18.22 µM, <1 to 12.61 µM and <1 to 9.25 µM for 24, 48 and 72 h respectively. The MDA-MB-231 cells were again the most susceptible to **2a-d** complexes with lower IC_{50} values, lower than1 µM.

<<Figure 2, 3, 4 and 5 are here>>

Figure 2, 3, 4 and 5 shows the dose-dependent antiproliferative effect of synthetic **(1a-d)** salts having chloride counter anions and synthetic complexes **(2a-d)** having silver

counteranions on DU-145, MCF-7, MDA-MB-231, and L-929 cells, respectively. A comparison of cytotoxicity between the cell lines identifies compounds as selective anticancer agents. **1a** salt did not display any activity toward any cell line tested even at a concentration of 20 µM. Whereas **1d** salts displayed similar activity in all the cell lines tested. This suggests that **1d** salts would display potentially poor selectivity in vivo. In contrast, **1b** salts displayed activity only against MDA-MB-231 cells. **1c** salts exert differences in cytotoxicity among all cell lines. Complexes **2c** and **2d** showed the greatest differences in activity between all cell line tested, **2a** and **2b** only displayed activity against DU-145 cells.

M. Napoli et. al. were synthesized, characterized and evaluated the antibacterial activity of five new silver complexes having bidentate N-heterocyclic carbene ligands [13]. They were used four ligands neutral, having an alcohol group on alkyl substituent of one of the two nitrogen atoms of the heterocycle [NHCeOH]. The last ligand having a alkoxide, is mono-anionic [NHCeO]. They were evaluated antibacterial activity for all the reported compounds, using a standard assay against a Gram negative (*Escherichia coli*) and a Gram positive (*Bacillus subtilis*) strain. The results of the study showed that all samples were not cytotoxic, whereas antibacterial activity, tested on *E. coli* and *B. subtilis,* complexes 1 and 10 have a MIC of 5 mg/ml. Thus, the complexes 1 and 10 have suggests that they can be very promising candidates for use as antibacterial compounds. The complexes 2 and 3 kill *E. coli* cells at a concentration of 5 mg/ml, but a higher concentration was necessary to inhibit the *B. subtilis* growth (50 mg/ml). M. Napoli et. al. reported that the synthesis and characterization of titanocenes compounds containing dialkyl ether groups appended to the Cp ligands, having different leaving groups on the metal [24]. All the compounds have been fully characterized by NMR, FT-IR and elemental analysis. Their cytotoxic activities have been evaluated on human breast cancer (MCF-7), human embryonic kidney (HEK-293) and murine macrophage (J774.A1) cells. IC_{50} value of the complex 3, which is the most-active complex, determined that 57 µM for MCF-7 cells. They were also reported that complex 2 more cytotoxic than complex 7 on MCF-7 cells. Complexes 2 and 7 have the same basic structure, differing only in the type of metal cation: in complex 2 it is titanium and in the complex 7 it is zirconium.

BK. Rana et. al have synthesized and characterized novel Au(I)− and Au(III)−NHC complexes [25]. In addition to the characterization of the complexes 2 and 3, their cytotoxicities were tested against human cancer cell lines, including HCT 116, HepG2, A549, and MCF7, and compared to that displayed by cisplatin. They were determined that Gold(I), complex 2 was more potent as an anticancer agent than 3 or cisplatin in four cell lines. IC_{50}

values of complex 2, 3 and cisplatin found to be 4.7 ± 0.8 µm, 6.2 ± 0 1.4 µm and 9.4 ± 1.0µm for MCF-7 cells, respectively. J. Dinda et. al. reported that synthesized and characterized using various spectroscopic techniques; starting ligand,1-methyl-2-pyridin-2-yl-2*H*-imidazo[1,5-*a*]pyridin-4-ylium chloride (**1 HCl**), three novel complexes [Ag(**1**)Cl] (**2**), [Au(**1**)Cl] (**3**) and [Au(**1**)Cl_3] (**4**) [26]. They have been studies cytotoxic activities of complexes **2**, **3**, and **4** against HepG2 (human hepatocellular carcinoma), HCT 116 (human colorectal carcinoma), A549 (human lung adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cells. They were reported that complex **3** exhibited a cytotoxicity that was similar to that of cisplatin towards all the four cancer cell lines tested. D. Lacopetta et. al. prepared a new four carbene complexes of silver and gold derived from 4,5- dichloro-1H-imidazole and 4,5-diphenyl-1H-imidazole [22]. They were stable to light and water, which makes them viable candidates for use as chemotherapeutic. They were reported that the synthesized complexes possess good antitumor activity MDAMB-231, HeLa and ISHIKAWA. Also, complexes showed better cytotoxic profile than cisplatin. The IC_{50} values of the most-active compound (AuL_4)were found to be (3.98±0.4) µm for MCF-7 cells and (6.02±0.7) µm for MDA-MB-231 cells. Wang et. el. reported that amino–NHC metal complex analogs inhibited the growth of MCF-7 and MDA-MB-231 cancer-cell lines. IC_{50} values of the most-active complexes reported that 6.47±0.07 µM and 4.50± 0.07 µm for MCF-7 cells and MDA-MB-23, respectively [38]. Liu et al. prepared a new series of NHC–gold halide complexes derived from 4,5-diarylimidazoles. Bromo[1,3-diethyl-4,5-bis(2-fluorophenyl)- 1,3-dihydro-2H-imidazol-2-ylidene]gold(I), showed most antiproliferative activity on breast cancer cell lines with IC_{50} values of (0.87±0.07) µm on MCF- 7 and (3.1±0.5) µm on MDA-MB-231 [39].

All complexes **(2a-d)** are all more cytotoxic in the cancer cell line (except DU-145 cell line for 2a and 2b) than the healthy cells (L-929; normal cells adipose from mouse). Complexes showing the greatest difference in cytotoxicity between cancer cells and normal cells. Complex **2a**; more than 5-fold more cytotoxic toward the MCF-7 and MDA-MB-231 cancer cells, **2b**; more than 4.68-fold more cytotoxic toward the MCF-7 and MDA-MB-231 cancer cells, **2c;** 2.54-fold, 3.46-fold and more than 12 fold more cytotoxic toward the DU-145, MCF-7 and MDA-MB-231 cancer cells, respectively and **2d;** 1.83-fold, 7.48-fold and more than 11 fold more cytotoxic toward the DU-145, MCF-7 and MDA-MB-231 cancer cells, respectively.

Some studies have indicated that the ancillary ligands play an important role in biological

properties of compounds, thus differences of ancillary ligands in complexes may create some difference in the biological activities [40,41]. Although pure metals are biologically inactive, metal cations have biological activity. It was observed that the activity of the silver cation was increased by binding the silver cation to different ligands which have biologically compatible properties [42-45].

It has been previously reported that imidazole-based ortho-, meta-, and para-xylyl linked salts and their respective Ag(I)-NHC complexes display *in vitro* anticancer activity against the HCT 116 cell line and that the allyl-substituted para-xylyl linked dinuclear Ag(I)-NHC complex had higher cytotoxicity [44]. Another study also showed that the benzimidazole-based para-xylyl linked iso-propyl-substituted complex demonstrated higher cytotoxicity whereas the ortho- as well as the meta-xylyl linked complexes demonstrated comparatively moderate activities [37]. Similarly, our results showed that para-methylbenzyl substituted complexes more cytotoxic than, -ortho and –meta linked complexes. This activity could be attributed to the cavity and size of the molecule. The molecules with para substitution were assumed to have a bigger cavity and larger size, as compared to the ortho and meta units which increases the exposure of *N*-heterocyclic carbene (NHC) rings.

4. CONCLUSIONS

In conclusion, we synthesized four novel unsymmetrically substituted NHC ligands (**1a-d**) and their Ag(I) complexes (**2a-d**). Ag(I)-NHC complexes were found to display relatively high activity against cancer cell growth, as compared their respective ligands. These results supports the conclusion that silver cations are critical for the anti-cancer activity of these novel compounds. The higher cytotoxicity of Ag(I)-NHC complexes might be due to the deposition of silver cations in the membrane of cells and the organelles and thus may cause toxicity by interfering with cellular respiration and metabolism of biomolecules. The findings of the present work support the conclusion that the para-methyl linked compounds are more active compared to the complexes with ortho- and meta- compartments. In addition, the ortho-methyl substituted compound 2a have more cytotoxic activity than the ortho-chloro substituted compound 2d.

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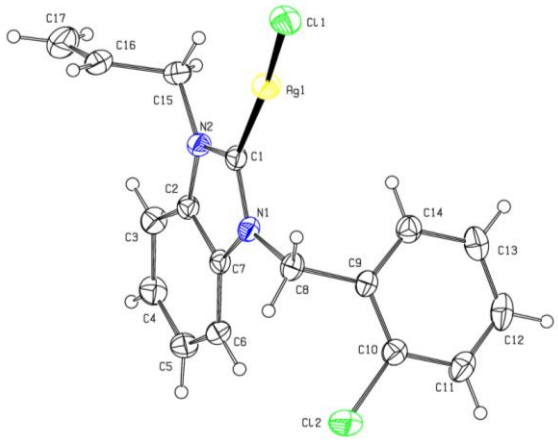


Figure 1: PLATON drawing of the **2d** compound with the atomic numbering scheme. Displacement ellipsoids are drawn at the 50 % probability level. The H-atoms are shown as small circles of arbitrary radii.

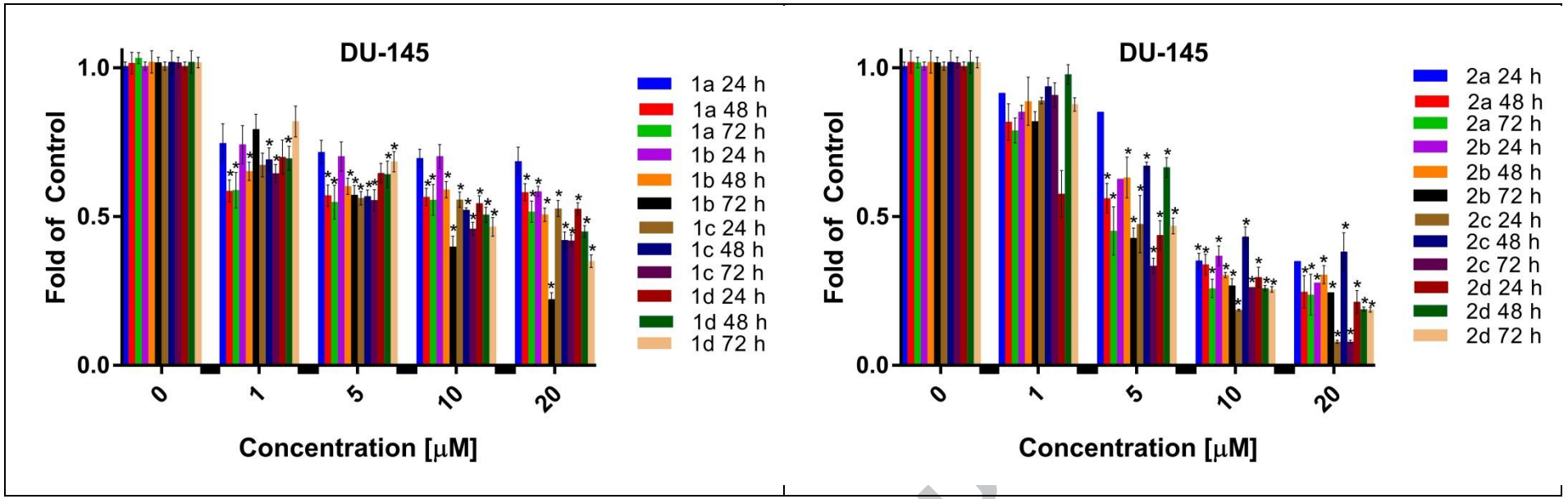


Figure 2. Effect of salts and complexes on MTT staining in DU-145 cells. DU-145 cells were treated with 1, 5, 10 and 20 μM of salts (**1a-d**) and Ag-complexes (**2a-d**) for 24, 48 and 72 h. Controls were cells treated with DMSO. Data are representative of the mean of three separate experiments done in triplicate, and are reported at the SEM. (* $p<0.0001$ vs control).

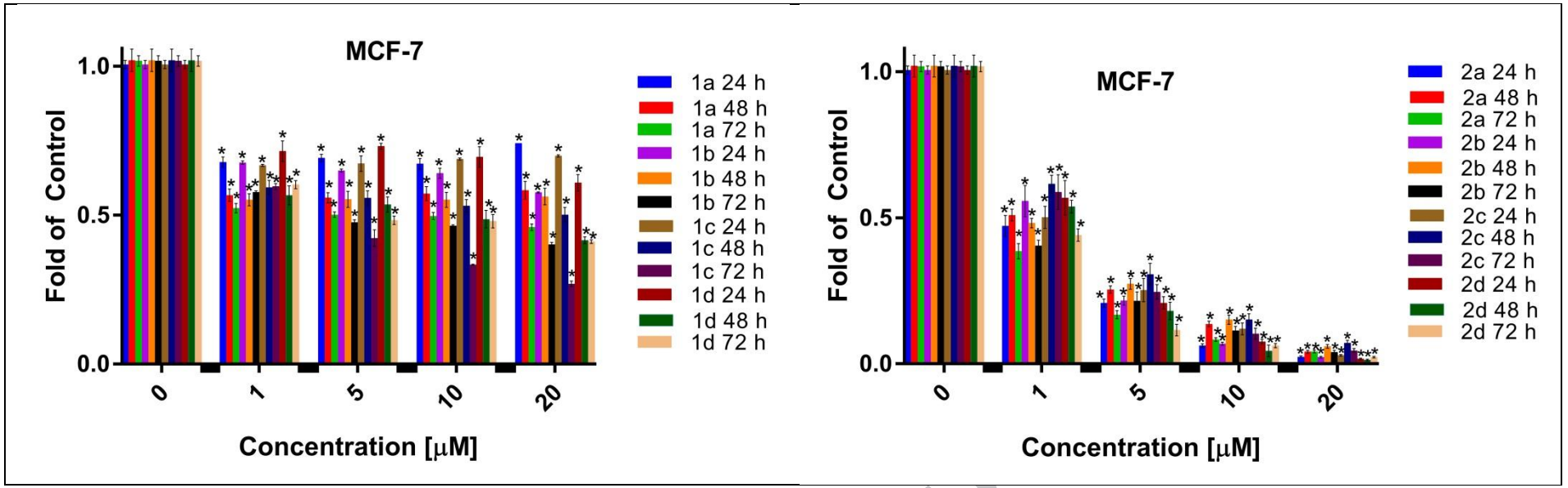


Figure 3. Cytotoxicity as determined by the MTT assay. MCF-7 cells treated with 1, 5, 10 and 20 μM of salts (**1a-d**) and Ag-complexes (**2a-d**) for 24, 48 and 72 h. DMSO treated cells were used as vehicle control. Data are representative of the mean of three separate experiments done in triplicate (± SEM). (* $p < 0.0001$ vs control).

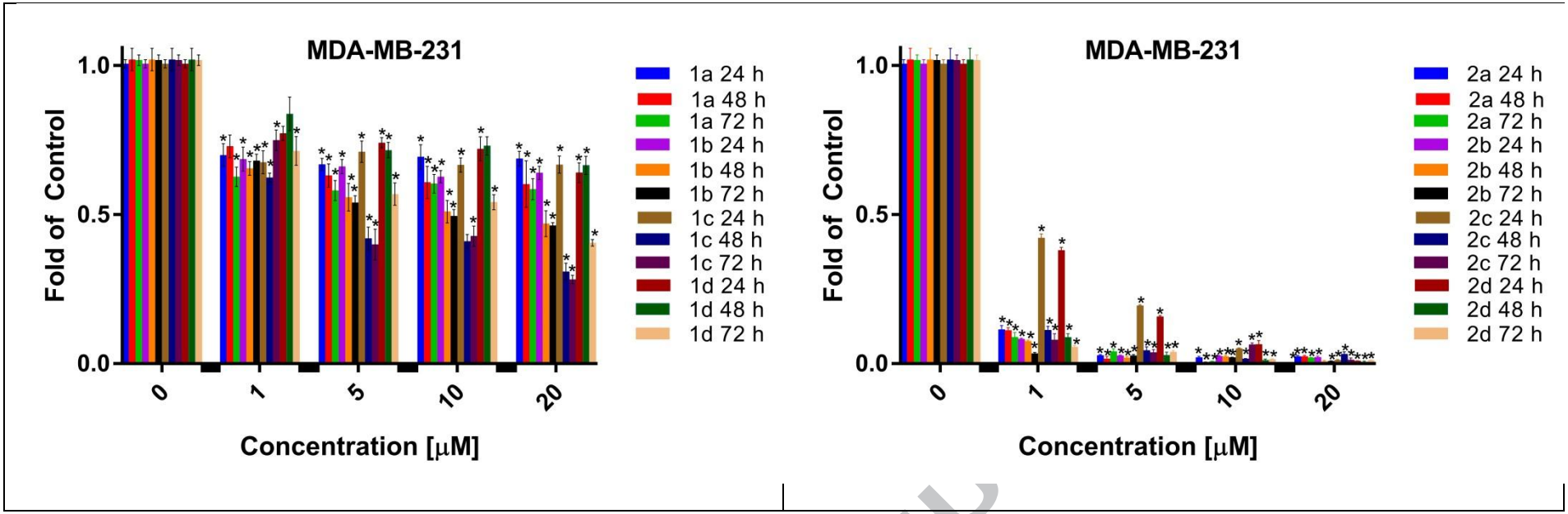


Figure 4. Cytotoxicity as determined by the MTT assay. MDA-MB-231 cells treated with 1, 5, 10 and 20 μM of salts (**1a-d**) and Ag-complexes (**2a-d**) for 24, 48 and 72 h. DMSO treated cells were used as vehicle control. Data are representative of the mean of three separate experiments done in triplicate (± SEM). (* $p < 0.0001$ vs control).

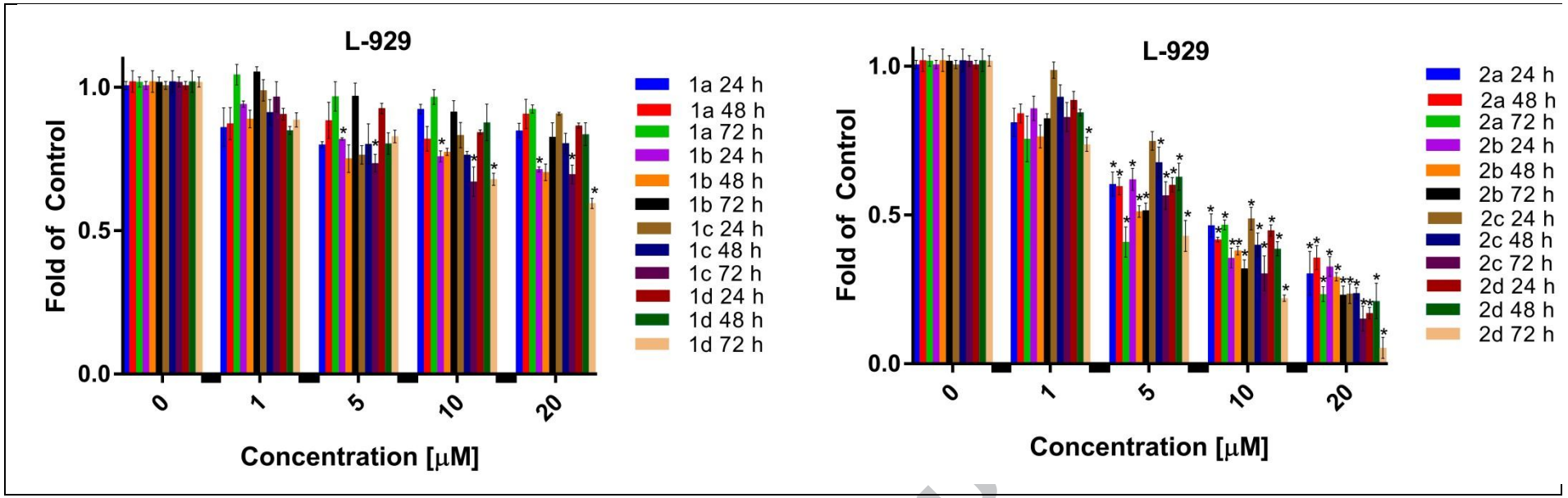
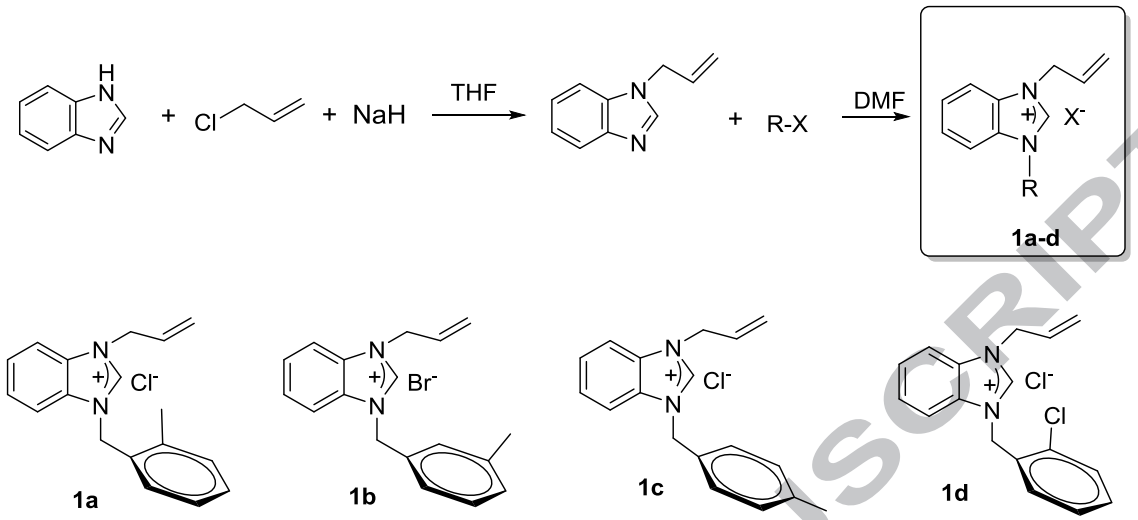
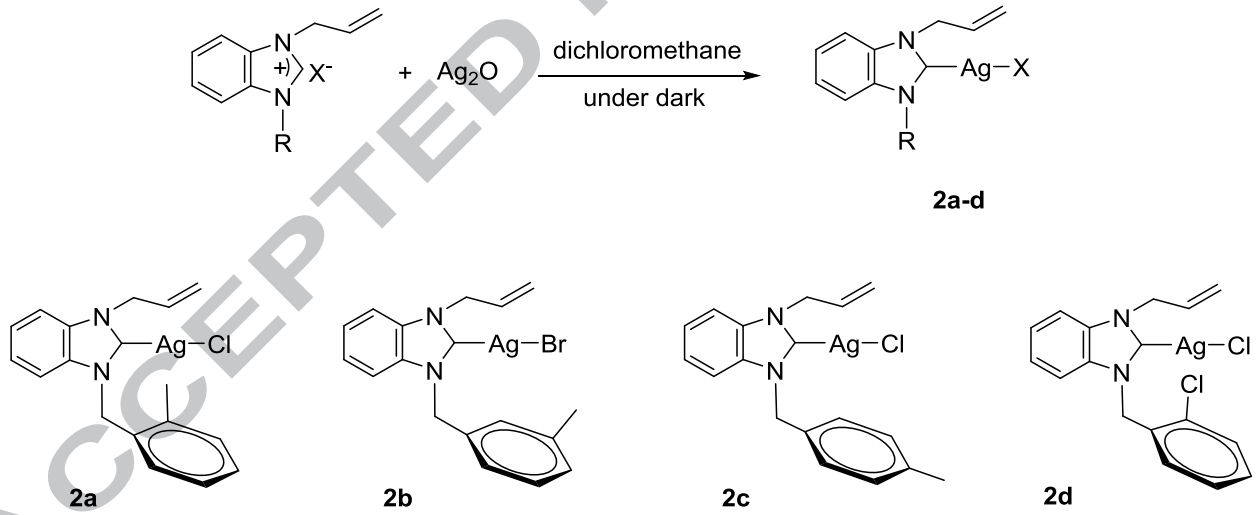


Figure 5. Cytotoxicity aas determined by the MTT assay. L-929 cells treated with 1, 5, 10 and 20 μM of salts (**1a-d**) and Ag-complexes (**2a-d**) for 24, 48 and 72 h. DMSO treated cells were used as vehicle control. Data are representative of the mean of three separate experiments done in triplicate (± SEM). (* $p < 0.0001$ vs control)



Scheme 1. Synthesis of benzimidazolium salts (**1a-b**)



Scheme 2. Synthesis of Ag-NHC complexes (**2a-d**)

Table 1. Selected geometric parameters (Å, °)

Ag1 C1 2.096(2)	Ag1 C*l*1 2.3554(7)	C*l*2 C10 1.742(3)
N1 C1 1.356(3)	N1 C7 1.390(3)	N1 C8 1.465(3)
N2 C1 1.347(3)	N2 C2 1.394(3)	

C1 Ag1 C*l*1 168.28(7)	C1 N1 C7 111.10(18)
C1 N1 C8 124.89(19)	C1 N2 C2 111.12(18)
C1 N2 C15 124.5(2)	C2 N2 C15 124.26(19)
N2 C1 N1 105.97(19)	N2 C1 Ag1 128.60(17)
N1 C1 Ag1 125.40(16)	

Table 2. Cytotoxic activities of ligands **(1a-d)** and complexes (**2a-d**) in selected human cancer and normal cells[a] in vitro (IC_{50}, μM[b]) after 48 h of incubation

Compounds	DU-145	MCF-7	MDA-MB-231	L-929[a]
		48 h		
1a	>20	>20	>20	>20
1b	>20	>20	5.17 ± 0.65	>20
1c	10.03 ± 0.40	13.61 ± 0.91	3.85 ± 0.71	>20
1d	20.00 ± 0.57	>20	>20	>20
2a	6.02 ± 0.30	<1	<1	5.08 ± 0.20
2b	5.16 ± 0.33	<1	<1	4.68 ± 0.15
2c	4.97 ± 0.25	3.64 ± 0.16	<1	12.61 ± 0.21
2d	6.52 ± 0.18	1.60 ± 0.17	<1	11.97 ± 0.24

[a] Non-cancer cells,.[b]Cell viability after treatment for 48h was determined by MTT staining as described in the Experimental section. Each IC_{50} value represents the mean ± SEM of three independent experiments.

Highlights

- Novel N-Heterocyclic Carbene Silver(I) Complexes
- Structural characterization by elemental analysis, FT-IR, ^{1}H NMR and ^{13}C NMR and X-ray crystallography techniques.
- Comparison of anticancer activities of ligands and Carbene Silver(I) complexes on prostate and breast cancer cells and L-929 non-cancer cells.
- Selectivity for human cancer cells and non-cancer cells