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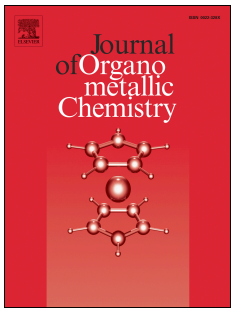

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Tetra *N*-heterocyclic carbene dinuclear silver(I) complexes as potential anticancer agents: Synthesis and *in vitro* anticancer studies

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ABSTRACT

Present study involves the synthesis and characterization of a newly designed tetrakis benzimidazolium salts, **1**-**5** and their respective tetra *N*-heterocyclic carbene (NHC) dinuclear silver(I) complexes, **6**-**10** with different terminal substituents that constructed from either cyclopentyl, benzyl, *n*-butyl, 2-methylene benzonitrile or *n*-decyl. These well characterized compounds were then tested for anticancer potential by determining their IC_{50} values and antiproliferative activity on HCT116, MCF-7 and HeLa cancer cell lines. Anticancer potential of these compounds was then determined by evaluating the mode of cytotoxicity of these novel compounds. Among the synthesized compounds, the proligand **5** and silver(I)-NHC complex, **10** having *n*-decyl substitution show selective antiproliferation activity against human colon cancer cell lines (HCT-116). All the complexes **6**-**10** and proligand **5** show moderate and strong antiproliferative activity against breast cancer and cervical cancer possibly by inhibiting colony formation and migration of cancer cells.

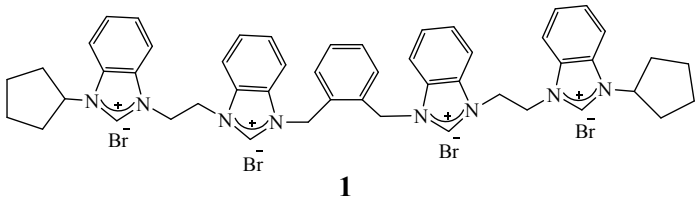
Keywords: Tetra *N*-heterocyclic carbene; Dinuclear silver(I) complexes; Antiproliferation; Colony formation.

1. Introduction

The recognition of the *N*-heterocyclic carbenes (NHC) as ligands in metal complexes by Wanzlick and Öfele[1-2] followed by the successful isolation of the free NHC by Arduengo[3], led to the complexation of NHC with a wide variety of the main group and the transition metals[4]. Out of different NHC metal complexes, the silver(I)-NHC complexes have contributed much to this field. Although this area has been extensively studied for the mono-NHC complexes, the introduction of poly-NHC complexes, which includes di-, tri-, tetra- and hexa- NHC bearing metal complexes, have further extended their research perspectives. To date, a diverse library of NHC complexes with different architectures, complexation pattern, denticity of ligand and number of metal centres have been successfully synthesized and evaluated for different kind of activities[5-6]. Among the poly-NHC complexes, the tri-, tetra- and hexa-NHC complexes have furnished a limited number to the literature owing to their complicated multistep synthesis[7-19].

In our efforts to contribute to the tetra-NHC literature, we have promulgated a successful synthesis of an open chain tetrakis benzimidazolium salt with a new architecture that serves as a precursor for the tetra NHC ligand, **1** and its respective silver(I) complex, **6** (Figure 1), through a new multistep designed scheme[20]. In the present work, we have extended the synthesis to obtain four new tetrakis benzimidazolium salts that are precursors for the tetra-NHC ligands to be linked with silver(I) ion (Scheme 1). The selection of silver(I) for complexation with NHC is based on the widespread applications offered by silver(I)-NHC complexes over the other metal complexes associated with NHC entity. The most striking applications are including transmetallation[21-24] and catalysis[25-27]. Additionally, owing to the

medicinal significance of silver metal, the silver(I)-NHC complexes are studied for their antimicrobial [28-30] as well as anticancer potential [31-35].



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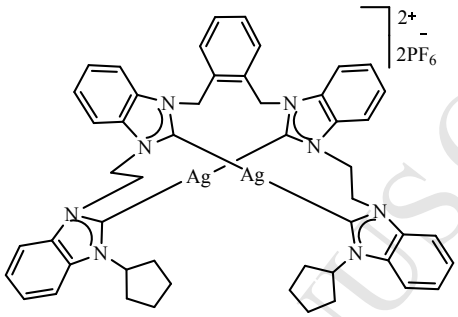

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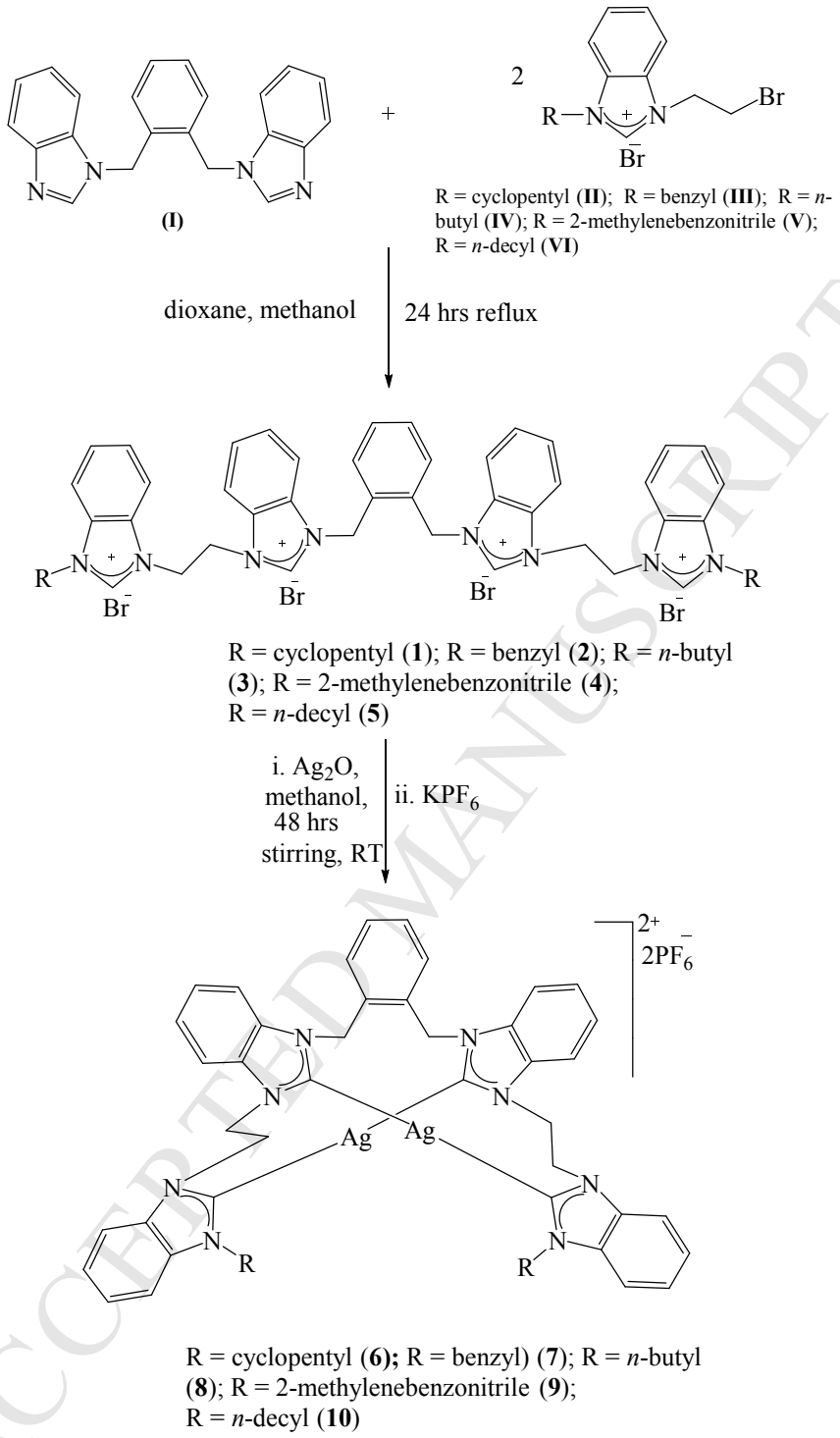
Figure 1: Structure of tetra-NHC proligand, **1** and the respective silver(I)-NHC complex, **6**[20].

The mono- and di-NHC silver(I) complexes were reported before to have a significance anticancer activity against different cancer cell lines[36]. Keeping in view the potential of silver(I)-NHC complexes as the emerging anticancer agents, the anticancer studies of the synthesized compounds against various cell lines were carried out. To the best of our knowledge, the tetra-NHC dinuclear silver(I) complexes have never been studied before for such an activity. All the synthesized silver(I)-NHC complexes and the respective proligands were studied for their anticancer potential against the HCT116, MCF-7 and the HeLa cell lines along with the exploration of mechanism for anticancer effect.



Scheme 1. Synthesis of tetrakis benzimidazolium salts (**1**-**5**) and the respective tetra NHC-dinuclear silver(I) complexes (**6**-**10**).

2. Experimental

2.1. Materials and Instruments

All the chemicals and solvents were of analytical grade and used as received. FTIR spectra were recorded on a Perkin Elmer 2000 spectrometer. ^{1}H- and ^{13}C-NMR spectral analysis was completed on Bruker 500 MHz spectrometer. Elemental analysis was carried on Perkin Elmer series II, 2400 microanalyzer. Melting points were taken using Stuart Scientific SMP-1 (UK) instrument.

2.2 Synthesis

The preparations of 1,2-bis(benzimidazol-1ylmethyl)benzene (**I**), 3-(2-bromoethyl)-1-cyclopentyl benzimidazolium bromide (**II**), proligand **1** and respective dinuclear silver(I) complex **6** are as previously reported[20].

*2.2.1 Synthesis of 3-(2-bromoethyl)-1-benzyl benzimidazolium bromide (**III**)*

N-benzyl benzimidazole (1.00 g, 4.80 mmol) was reacted with 1,2-dibromoethane (4 mL, 46 mmol) in neat. The reaction mixture was refluxed at 80°C for 8 hours. After completion of reaction time the product appeared as white crystalline salt. The excess 1,2-dibromoethane was removed by rotary evaporator, in which dichloromethane was then added to the solid residue and was filtered. The filtrate was left to evaporate in the fume cupboard and after evaporation of solvent the product was collected in crystalline form. Yield: 1.05 g (53%). M.p.: 205-207°C. Anal.Calc.for $C_{16}H_{16}Br_2N_2$: C, 48.48; H, 4.04; N, 7.07. Found: C, 48.24; H, 3.94; N, 6.77. FTIR (KBr) υ (cm^{-1}): 3136, 3025 (C_{arom}-H); 2966, 2884 (C_{aliph}-H), 1610, 1492, 1458 (C_{arom}=C_{arom}), 1561 (C_{arom}-N), 1263 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 3.85 (2H, -CH_2-Br, t, J = 5.0 Hz), 4.62 (2H, N-CH_2-, t, J = 5.0 Hz), 5.85 (2H, N-CH_2-Ar, s),

7.35-8.13 (9H, Ar-H, m), 10.01 (1H, NCHN, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 49.6 (CH_2-Br), 49.7 (N-CH_2), 58.5 (Ar-CH_2); 114.1, 126.5, 128.2, 128.9, 130.7, 131.5, 134.0 (Ar-C); 142.8 (NCHN).

*2.2.2 Synthesis of 3-(2-bromoethyl)-1-n-butyl benzimidazolium bromide (**IV**)*

Salt **IV** was prepared following the same procedure as that for salt **III** except that *N*-cyclopentylbenzimidazole was replaced with *N*-*n*-butylbenzimidazole (1.00 g, 5.70 mmol). Salt **IV** was collected as colorless crystals. Yield: 1.20 g (58%). M.p.: 115-118°C. Anal.Calc.for $C_{13}H_{18}Br_2N_2.H_2O$: C, 41.05; H, 5.26; N, 7.36. Found: C, 41.17; H, 5.04; N, 7.36. FTIR (KBr) υ (cm^{-1}): 3124, 3067 (C_{arom}-H); 2960, 2865 (C_{aliph}-H), 1618, 1481, 1448 (C_{arom}=C_{arom}), 1561 (C_{arom}-N), 1260 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.93 (3H, CH_3, t, *J* = 5.1 Hz), 1.34 (2H, CH_3-CH_2-, sext, *J* = 5.0 Hz), 1.91 (2H, CH_2-*CH_2*-CH_2, m), 4.07 (2H, -CH_2-N, t, *J* = 5.2 Hz,), 4.57 (2H, -CH_2-*CH_2*Br, t, *J* = 5.0 Hz), 5.02(2H, *CH_2*-CH_2Br, t, *J* = 6.0 Hz), 7.72-8.20 (4H, Ar-H, m), 10.04 (1H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 13.3 (CH_3); 18.9, 30.4, 31.1, 46.5, 47.9 (CH_2); 114.0, 126.7, 126.9, 130.9 (Ar-C); 142.6 (NCHN).

*2.2.3 Synthesis of 3-(2-bromoethyl)-1-(2-methylene benzonitrile) benzimidazolium bromide (**V**)*

Salt **V** was prepared following the same procedure as that for salt **III** except that *N*-cyclopentyl benzimidazole was replaced with *N*-(2-methylene) benzonitrile benzimidazole (1.70 g, 7.00 mmol). Salt **V** was collected as colorless crystals. Yield: 1.40 g (50%). M.p.: 127-129°C. Anal.Calc.for $C_{17}H_{14}Br_2N_3.H_2O$: C, 46.46; H, 3.87; N, 9.56. Found: C, 46.09; H, 3.88; N, 9.14. FTIR (KBr) υ (cm^{-1}): 3109, 3025 (C_{arom}-H); 2979 (C_{aliph}-H), 2221(C≡N), 1641, 1610, 1485 (C_{arom}=C_{arom}), 1553 (C_{arom}-N), 1267 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 4.08 (2H, CH_2-Br, t, *J* = 5.0 Hz), 5.07 (2H, N-*CH_2*-CH_2, t, *J* = 5.2 Hz), 6.12 (2H, CH_2-

Ar, s), 7.44-7.78 (4H, H-Ar, m), 7.96-8.27 (4H, H-ArCN, m), 10.01 (1H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 31.0, 48.5, 54.9 (CH_2); 111.0, 114.3 (Ar-C); 116.9 (CN); 127.1, 128.9, 129.7, 131.0, 133.8, 134.1, 136.9 (Ar-C); 143.7 (NCHN).

*2.2.4 Synthesis of 3-(2-chloroethyl)-1- n-decyl benzimidazolium bromide (**VI**)*

Salt **VI** was prepared following the same procedure as that for salt **III** except that *N*-cyclopentylbenzimidazole was replaced with *N*-*n*-decylbenzimidazole (1.40 g, 5.40 mmol) and involves the use of 1-bromo-2-chloroethane (3.80 mL, 46 mmol). Salt **VI** was collected as colorless crystals. Yield: 1.20 g (50%). M.p.: 124-144°C. Anal.Calc.for $C_{19}H_{30}BrClN_2.H_2O$: C, 54.35; H, 7.62; N, 6.67. Found: C, 54.76; H, 7.35; N, 6.55. FTIR (KBr) υ (cm^{-1}): 3125, 3025 (C_{arom}-H); 2960, 2874 (C_{aliph}-H), 1618, 1482, 1453 (C_{arom}=C_{arom}), 1560 (C_{arom}-N), 1260 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.82 (3H, CH_3, t, *J* = 7.0 Hz), 1.19-1.28 (14H, 7 × -CH_2-, m), 1.90-1.93 (2H, -CH_2-, m), 4.09 (2H, N-CH_2-, t, *J* = 6.2 Hz), 4.54 (2H, CH_2-Cl, t, *J* = 5.2 Hz), 5.07 (2H, N-*CH_2*-CH_2-Cl, t, *J* = 5.0 Hz), 7.70-8.21 (4H, H-Ar, m), 10.13 (1H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 13.7 (CH_3), 21.8, 25.5, 28.4, 30.9, 31.0, 42.4, 46.9, 47.9, 48.1, 49.2, 58.5 (CH_2), 113.5, 127.0, 131.1 (Ar-C); 142.2 (NCHN).

*2.2.5 Synthesis of benzyl substituted tetrakis benzimidazolium bromide salt (**2**)*

The 1,2-bis(benzimidazol-1-ylmethyl)benzene (0.4 g, 1.3 mmol) was dissolved in 30 mL of 1,4-dioxane on heating and then salt **III** (1.0 g, 2.5 mmol) was added to the reaction mixture, 2-3 mL of methanol was added in order to completely dissolve the reactants. The reaction mixture was refluxed with stirring for 24 hours. The product settled down as sticky white solid which was filtered, washed with fresh 1,4-dioxane and then with dichloromethane and diethyl ether. The salt **2** was collected off as white powder. Yield: 0.80 g (55%). M.p.: 257-261°C. Anal.Calc.for $C_{54}H_{50}Br_4N_8$: C, 56.44; H, 4.35; N, 9.91. Found: C, 56.88; H, 4.49;

N, 10.06. FTIR (KBr) υ (cm^{-1}): 3155, 3102 (C_{arom}-H), 1618, 1486, 1431 (C_{arom}=C_{arom}), 1566 (C_{arom}-N), 1279 (C_{aliph}-N).^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 5.26 (8H, 2 × CH_2-CH_2, s), 5.77 (4H, 2 × CH_2-Ar, s), 5.98 (4H, 2 × CH_2-Ar, s), 7.14-8.00 (30H, Ar-H, m), 10.10 (2H, NCHN, s), 10.20 (2H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 45.7, 47.8, 50.1, 66.3 (CH_2); 111.5, 113.4, 114.0, 118.4, 123.4, 126.9, 128.3, 128.9, 129.4, 131.7, 133.5, 134.8 (Ar-C); 143.3, 143.7 (NCHN).

*2.2.6 Synthesis of n-butyl substituted tetrakis benzimidazolium bromide salt (**3**)*

Salt **3** was prepared following the same procedure as that for salt **2** except that salt **III** was replaced by **IV** (1.3 g, 3.6 mmol) and 1,2-bis(benzimidazol-1-ylmethyl)benzene (0.60 g, 1.80 mmol). The salt **3** was collected as off white powder. Yield: 1.02 g (52%). M.p.: 251-255°C. Anal.Calc.for $C_{48}H_{54}Br_4N_8$: C, 54.25; H, 5.09; N, 10.55. Found: C, 54.44; H, 5.22; N, 10.04. FTIR (KBr) υ (cm^{-1}): 3162, 3105 (C_{arom}-H), 2964, 2877 (C_{aliph}-H), 1618, 1490, 1458 (C_{arom}=C_{arom}), 1565 (C_{arom}-N), 1279 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.87 (6H, 2 × -CH_3, t, J = 7.0 Hz), 1.22 (4H, 2 × CH_2-CH_2-CH_3, m), 1.75 (4H, 2 × CH_2-CH_2-CH_2, m), 4.43 (4H, 2 × N-CH_2-CH_2, t, J = 7.5 Hz), 5.22 (8H, 2 × CH_2-CH_2-, s), 6.11 (4H, 2 × CH_2 benzylic, s), 7.04-8.11 (20H, Ar-H, m), 10.04 (2H, NCHN, s), 10.09 (2H, NCHN, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 13.4 (CH_3); 18.9, 30.4, 45.7, 46.5, 47.8 (CH_2); 113.0, 113.8, 114.2, 126.8, 128.4, 130.9, 131.1, 131.7 (Ar-C); 142.8, 143.6 (NCHN).

*2.2.7 Synthesis of 2-methylenebenzonitrile substituted tetrakis benzimidazolium bromide salt (**4**)*

Salt **4** was prepared following the same procedure as that for salt **2** except that salt **III** was replaced by **V** (0.80 g, 1.90 mmol) and 1,2-bis(benzimidazol-1-ylmethyl)benzene (0.30 g, 1.07 mmol). The salt **4** was collected as off white powder. Yield: 0.65 g, (55%). M.p.: 247-251°C. Anal.Calc.for $C_{56}H_{48}Br_4N_{10}$: C, 56.95; H, 4.07; N, 11.86. Found: C, 56.55; H, 4.32; N, 11.47. FTIR (KBr) υ (cm^{-1}): 3157, 3098 (C_{arom}-H), 2229(C≡N), 1614, 1490, 1446

(C_{arom}=C_{arom}), 1567 (C_{arom}-N), 1282 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 5.21 (8H, 2 × CH_2-CH_2, m), 5.99 (8H, 4 × CH_2-Ar, m), 7.34-8.00 (28H, Ar-H, m), 9.96 (2H, NCHN, s), 10.01 (2H, NCHN, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 45.6, 47.8, 49.1 (CH_2); 111.6, 112.7, 113.7, 116.5, 127.7, 129.5, 129.9, 130.1, 131.1, 133.7, 134.9 (Ar-C); 141.5, 142.0 (NCHN).

*2.2.8 Synthesis of n-decyl substituted tetrakis benzimidazolium bromide salt (**5**)*

Salt **5** was prepared following the same procedure as that for salt **2** except that salt **III** was replaced by **VI** (1.3 g, 3.2 mmol) and 1,2-bis(benzimidazol-1-ylmethyl)benzene (0.54 g, 1.6 mmol). The salt **5** was collected as off white powder. Yield: 0.9 g, (50%). M.p.: 275-279°C. Anal.Calc.for $C_{60}H_{78}Br_2Cl_2N_8$: C, 63.04; H, 6.83; N, 9.80. Found: C, 62.89; H, 6.52; N, 9.55. FTIR (KBr) υ (cm^{-1}): 3175, 3109 (C_{arom}-H); 2964, 2880 (C_{aliph}-H), 1618, 1490, 1453 (C_{arom}=C_{arom}), 1565 (C_{arom}-N), 1275 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.85 (6H, 2 × CH_3, t, *J* = 7.0 Hz), 1.23-1.26 (28H, 14 × -CH_2-, m), 1.78 (4H, 2 × -CH_2-, m), 4.43 (4H, 2 × N-CH_2-, t, *J* = 7.5 Hz), 5.15-5.20 (8H, 2 × CH_2-CH_2, m), 5.99 (4H, 2 × CH_2-Ar, s), 7.17-8.31 (20H, Ar-H, m), 9.82 (2H, NCHN, s), 10.14 (2H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 13.9 (CH_3); 22.0, 25.6, 28.4, 31.2, 45.5, 45.8, 46.7, 47.8, 66.3 (CH_2); 110.8, 114.2, 121.8, 126.8, 129.2, 131.6, 133.7, 135.5 (Ar-C); 142.8, 144.2 (NCHN).

*2.2.9 Synthesis of benzyl substituted tetra NHC dinuclear silver(I) complex (**7**)*

Salt **2** (0.54 g, 0.47 mmol) was dissolved in 80 mL of methanol and silver oxide (0.44 g, 1.90 mmol) was added to it, the reaction mixture was stirred at room temperature for 2 days. The reaction was carried out in a round bottom flask wrapped with aluminium foil in order to exclude light. After completion of reaction time the product was separated from the insolubles like AgBr and unreacted Ag_2O by filtering through a column of celite. The resulted silver(I) complex bromide was subjected to metathesis using potassium

hexafluorophosphate (0.35 g, 1.90 mmol). The mixture was stirred for 2 hours and the resulting precipitates were filtered then washed with distilled water and left to dry at room temperature. The dinuclear silver(I) complex **7** was collected as white powder. Yield: 0.32 g (52%). M.p.: 225-229°C. Anal.Calc.for $C_{54}H_{46}Ag_2F_{12}N_8P_2$: C, 49.39; H, 3.51; N, 8.54. Found: C, 49.43; H, 3.14; N, 8.86. FTIR (KBr) υ (cm^{-1}): 3067, 3033 (C_{arom}-H); 2938 (C_{aliph}-H), 1610, 1481, 1454 (C_{arom}=C_{arom}), 1271 (C_{aliph}-N). ^{1}H NMR (500 MHz, Acetonitrile-d_3) δ ppm: 5.26 (8H, 2 × CH_2-CH_2, s), 5.55 (4H, 2 × CH_2-Ar, s), 6.54 (4H, 2 × CH_2-Ar, s), 7.10-8.21 (30H, Ar-H, m). ^{13}C NMR (125 MHz, Acetonitrile-d_3) δ ppm: 45.6, 47.7, 50.7 (CH_2); 112.3, 113.6, 113.8, 125.1, 127.7, 128.3, 129.0, 129.6, 132.9, 133.0, 133.4, 134.9 (Ar-C); 188.6 (C-Ag, br d).

*2.2.10 Synthesis of n-butyl substituted tetra NHC dinuclear silver(I) complex (**8**)*

Complex **8** was prepared following the same procedure as that for complex **7** except that salt **2** was replaced by **3** (0.50 g, 0.47 mmol) and silver oxide (0.43 g, 1.90 mmol). The dinuclear silver(I) complex **8** was collected as white powder. Yield: 0.34 g (59%). M.p.: 219-223°C. Anal.Calc.for $C_{48}H_{50}Ag_2F_{12}N_8P_2$: C, 46.30; H, 4.02; N, 9.00. Found: C, 46.54; H, 4.01; N, 9.24. FTIR (KBr) υ (cm^{-1}): 3159, 3067 (C_{arom}-H), 2949, 2873 (C_{aliph}-H), 1614, 1481, 1462 (C_{arom}=C_{arom}), 1270 (C_{aliph}-N). ^{1}H NMR (500 MHz, Acetonitrile-d_3) δ ppm: 0.74 (6H, 2 × -CH_3, t, J = 7.0 Hz), 1.22 (4H, 2 × CH_2-CH_2-CH_3, m), 1.64 (4H, 2 × CH_2-CH_2-CH_2, m), 5.12 (4H, 2 × N-CH_2-CH_2, t, J = 7.5 Hz), 5.30 (8H, 2 × CH_2-CH_2-, s), 5.74 (4H, 2 × CH_2 benzylic, s), 7.04-8.31 (20H, Ar-H, m).^{13}C NMR (125 MHz, Acetonitrile-d_3) δ ppm: 12.3 (CH_3); 18.7, 30.3, 45.4, 47.1, 48.3, 50.1 (CH_2); 117.1, 123.9, 126.7, 127.2, 130.5, 133.0, 134.8, 135.0 (Ar-C); 188.8 (C-Ag, br d).

*2.2.11 Synthesis of 2-methylenebenzonitrile substituted tetra NHC dinuclear silver(I) complex (**9**)*

Complex **9** was prepared following the same procedure as that for complex **7** except that salt **2** was replaced by **4** (0.50 g, 0.40 mmol) and silver oxide (0.41 g, 1.60 mmol). The dinuclear silver(I) complex **9** was collected as white powder. Yield: 0.28 g (52%). M.p.: 231-235°C. Anal.Calc.for $C_{56}H_{44}Ag_2F_{12}N_{10}P_2$: C, 49.74; H, 3.23; N, 10.28. Found: C, 50.0; H, 2.92; N, 10.32. FTIR (KBr) υ (cm^{-1}): 3117, 3063 (C_{arom}-H), 2224(C≡N), 1610, 1477, 1450 (C_{arom}=C_{arom}), 1286 (C_{aliph}-N). ^{1}H NMR (500 MHz, Acetonitrile-d_3) δ ppm: 5.18 (8H, 2 × CH_2-CH_2, m), 5.74 (8H, 4 × CH_2-Ar, m), 7.01-8.18 (28H, Ar-H, m). ^{13}C NMR (125 MHz, Acetonitrile-d_3) δ ppm: 45.1, 47.6, 50.2 (CH_2); 110.1, 110.7, 111.2, 112.1, 116.6, 124.2, 124.7, 125.1, 126.6, 128.4, 128.7, 130.1, 132.5, 133.3 (Ar-C); 188.7 (C-Ag, br d).

*2.2.12 Synthesis of n-decyl substituted tetra NHC dinuclear silver(I) complex (**10**)*

Complex **10** was prepared following the same procedure as that for complex **7** except that salt **2** was replaced by **5** (0.50 g, 0.44 mmol) and silver oxide (0.41 g, 1.60 mmol). The dinuclear silver(I) complex **10** was collected as white powder. Yield: 0.33 g (53%). M.p.: 169-198°C. Anal.Calc. for $C_{60}H_{74}Ag_2F_{12}N_8P_2$: C, 51.01; H, 5.24; N, 7.93. Found: C, 51.38; H, 4.98; N, 7.58. FTIR (KBr) υ (cm^{-1}): 3163, 3102 (C_{arom}-H); 2972, 2880 (C_{aliph}-H), 1614, 1487, 1446 (C_{arom}=C_{arom}), 1271 (C_{aliph}-N). ^{1}H NMR (500 MHz, Acetonitrile-d_3) δ ppm: 0.68 (6H, 2 × CH_3, t, J = 7.0 Hz), 0.80-1.32 (28H, 14 × -CH_2-, m), 1.74 (4H, 2 × -CH_2-, m), 4.68 (4H, 2 × N-CH_2-, t, J = 7.5 Hz), 5.15-5.45 (8H, 2 × CH_2-CH_2, m), 6.12 (4H, 2 × CH_2-Ar, s), 6.99-7.98 (20H, Ar-H, m). ^{13}C NMR (125 MHz, Acetonitrile-d_3) δ ppm: 13.1 (CH_3); 22.0, 28.6, 28.8, 29.1, 29.8, 31.3, 31.4, 48.5, 49.9, 54.1 (CH_2); 111.5, 112.0, 124.0, 124.2, 124.5, 124.6, 133.1 (Ar-C).

2.3 *In vitro* anticancer studies

2.3.1 Preparation of cell culture

First step in the preparation is the growth of HCT 116, MCF-7, human cervical cancer line HeLA cell line and human endothelial cell line EA.hy926 cell lines under incubation condition. Only those cells were selected for plating purpose which reached about 70-80% level of confluency. Medium used in the plates previously was aspirated and cells used were washed with phosphate buffer saline solution for 2-3 times. After washing with phosphate buffer saline solution, trypsin was added evenly on the cell surfaces. Cells were incubated for 1 minute at a temperature of 37°C. Cell segregation was facilitated by simply tapping the flask containing the cells and was observed under the microscope. A volume of 5 mL of fresh media was added to inhibit the trypsin activity. After counting, a final density of 2.5×10^5 cell/mL which was later inoculated to the wells of 96 well plate (100µL cells/well). These seeded plates were then incubated at standard atmospheric conditions for cell culture.

2.3.2 MTT assay for cell viability on different cancer cell lines

MTT assay was performed as reported[37]. Volumes of 100 µL of the cells were seeded in all wells of 96 wells microplate and microplate was incubated overnight for cell attachment under CO_2 incubator. Later on, a volume of 100 µL of each test substance was added in each well and plate was labelled accordingly. Different dilutions of the test substance were prepared to see dose dependent response. After the addition of test substance into the plate containing cancer cells, plate was incubated at 37 °C with 5% CO_2 environment for 72 h. After 72 h of treatment period, a volume of 20 µL of MTT reagent was added into each well and again incubated for 4 h. After this period of incubation, 20µL of DMSO (MTT lysis solution) was added in each well. Plates were further incubated for 5 minutes under same

environment of incubation. Finally, absorbance was taken at 570 nm and 620 nm by using microplate reader (Epoch, BioTek, USA). Data was analyzed for the cell viability and percentage inhibition of proliferation of test substances. The results were presented as percent viability compared to the negative control (mean ± SD, n = 3)[38].

2.3.3 Study of mode of cytotoxicity for tetra-NHC dinuclear silver(I) compounds

Screening for IC_{50} values and percentage inhibition of cell proliferation of all proligands and complexes of the tetra-NHC dinuclear Ag(I) series was completed on human colorectal tumor (HCT 116) cell lines, human breast cancer cell line (MCF-7), human cervical cancer line (HeLa cell line) and human endothelial cell line (EA.hy926 cell line). The most active proligand and its respective Ag(I)-NHC complex were selected for mechanistic study using colony formation assay and cell migration assay. The selectivity indices (SI) for selected compounds were also calculated by comparing the IC_{50} values of the respective compounds in cancerous as well as normal cell lines. The compounds were considered selectively cytotoxic, when the SI values were greater than 2[39-41]. The selectivity indexes were calculated according to formula:

$$\text{selectivity index} = \frac{IC_{50} \text{ in normal cell line}}{IC_{50} \text{ in cancerous cell line}}$$

2.3.3(a) Cell migration assay of MCF-7 cells treated with selected proligand and respective tetra-NHC dinuclear silver(I) complex

The *in vitro* assay, involves scratching of monolayer cells artificially to assess the effectiveness of chemicals, to deny cells from migration and to close the created wound[42]. A volume of 2 mL of culture medium (DMEM) containing MCF-7 cell line at a density of 2×10^5 cells/ mL was pipetted into each well of a 6-well plate. Then the plate was incubated

for 48 hrs under the culture conditions of a humidified incubator at 37ºC and 5% CO_2 to achieve absolute confluent monolayer cells. After that, the middle of the monolayer was handily scraped to create a straight scratch using a sterile 200µL pipette tip; the scratches were almost of similar size. The media was discarded carefully, and the plate was rinsed twice with PBS to remove the debris, then the consumed medium was replenished with fresh DMEM containing the tetra NHC proligand (6.25 and 12.5 µg/mL) and its respective Ag(I) complex (2.5 and 5 µg/mL) at two different concentrations which were selected on the basis of % inhibition of proliferation and IC_{50} values. Subsequently, images were acquired for the wounds at zero time by using a fluorescent microscope at × 40 magnifications. Plates were re-incubated with microscopic observations at different time intervals for the cell movement towards each other surrounding the wound. After 6 and 12 hrs, the cells were further imaged. Finally, the images were analyzed by measuring the distance between the cells of both sides of the scratch using Image J software. The measured distances were reported as the percentage of inhibition of migration in comparison to the mean distance for the negative control which was calculated according to formula:

$$\% \text{ Inhibition of migration} = [1 - \left(\frac{Ds}{Dc}\right)] \times 100$$

Where:

Ds = Distance travelled by cells treated with particular proligand and its respective complex.

Dc = Distance travelled by negative control cells.

The results were expressed as mean ± SD, (n = 3).

2.3.3(b) Colony formation assay on MCF-7 cell line treated with selected proligand and respective tetra-NHC silver(I) complex

This assay was designed to evaluate the ability of chemicals to suppress the reproductive potential of cell division and colonization after treatment[43]. The single-cell

suspension of breast cancer cell line (MCF-7) was diluted with DMEM culture medium. In order to obtain the seeding density of 500 cells/ mL, a volume of 2 mL was pipetted into each well of a sterile 6-well plate (Coster Corning, USA). Later the plate was incubated in a humidified atmosphere at 37ºC and 5% CO_2 for 12 hrs to permit the attachment of the cells and before the subdivision of the cells started. Subsequently, the old medium was aspirated carefully and replenished with a fresh medium containing the active tetra-NHC proligand **5** (6.25, 12.5 and 25 µg/mL) and its respective dinuclear silver(I) complex, **10** (2.5, 5 and 10 µg/mL) at three different concentrations mL which were selected on the basis of percentage inhibition of proliferation and IC_{50} values. Tamoxifen (25 and 10 µg/mL) was used as a positive control and the cells were treated with DMEM as a negative control of the experiment. Incubation was continued for 48 hrs then the medium was discarded and the wells were rinsed with PBS to remove the residue of the treatment and cell debris. A volume of 2 mL/well of fresh DMEM was added and repeatedly replenished every 3-4 days with routine microscopic monitoring until sufficiently large colonies of at least 20-25 cells were produced in the untreated wells. This process may take 10-12 days. Eventually, after aspiration of the medium, the wells were rinsed with PBS; colonies were fixed with 4% para-formaldehyde and then stained with 0.2% of crystal violet (Sigma-Aldrich, USA). Later the plate was washed with water to remove the excess dye. Finally, the colonies of more than 25 cells were counted manually under a dissecting microscope (Motic, Taiwan).

Calculations for surviving fraction (SF%) were done by the following:

$$SF\% = \frac{\text{no. of colonies of treated wells}}{(\text{no. of seeded cells} \times \text{PE})} \times 100$$

While plating efficiency (PE) was calculated by the following formula:

$$PE\% = \frac{\text{no. of colonies in negative control}}{\text{no. of seeded cells}} \times 100$$

2.3.4 Statistical analysis

The percentage inhibition of proliferation data was analysed for significance by two-way ANOVA and then compared by Bonferroni tests using GraphPad Prism Software, Version 5.01. One-way ANOVA was performed to study the overall mean percentage of inhibition of proliferation, cell migration and colony formation assays. The results presented are mean ± SD and were considered significant at $p < 0.05$.

3. Results and discussion

In the preparation of metal complexes containing tetra-NHC, we propose that the molecular structure for complexes **6**-**10** is somewhat similar to that reported structure of **5**[20], with the only different being the different terminal substituents moiety. While the crystal structure of **5** was isolated before, attempts to obtain crystals of complexes **6**-**10** were unsuccessful even under various crystallization means. However, based on all spectroscopic data (*vide infra*), the molecular arrangement for the later are agreeable to be that similar to **5**. All complexes is arranged in a twisted fashion with the single ligand has coordinated with two silver(I) cations (Scheme 1)[20]. Both silver(I) ions in each complexes are in linear geometry with the presence of two hexafluorohosphate anions in the lattice balances the entire structure.

3.1 *In vitro* anticancer activities of tetra-NHC dinuclear silver(I) complexes and their respective proligands against the human colon cancer (HCT116) cell line

The increased activity of Ag(I)-NHCs have been attributed to their increased stability which allows the release of silver(I) ions at a slower rate thereby increasing their efficacy[44]. Our previous reports indicated that the increasing of carbon chain length on wingtip of mono- and di-NHC silver(I) complexes increases the lipophilicity of compounds which resulted in increasing the cytotoxicity against human colon cancer cell line[37].

In context to the above findings, present study adopts a different approach by designing and synthesizing macromolecules containing tetra-NHC dinuclear silver(I) complexes. Keeping in view the potential benefits to toxicity ratio, number of silver(I) centres was kept limited while NHCs centres were increased by changing the wingtips on proligands and complexes. These macromolecules were tested against panel of different cancer cell lines and the most active compounds were further evaluated for the mode of cytotoxicity of these compounds by using different assays. It was hypothesized that greater steric hindrance around silver(I) ion in tetra-NHC dinuclear silver(I) complexes may result in stabilization, rendering the compounds to have better cytotoxicity. Initial screening was done by calculating the IC_{50} values and it was interesting to know that all the synthesized compounds were inactive on human colon cancer cell lines except for complex **7**, proligand **5** and respective complex **10** (Table 1).

Table 1: IC_{50} values of the proligands, **1**-**5** and respective complexes, **6**-**10** on HCT 116 using the standard 5-FU (IC_{50} = 10.2 µM).

Proligands	IC_{50} (µM)	NHC-Ag(I) complexes	IC_{50} (µM)
1	NA	**6**	NA
2	NA	**7**	85.42±5.25
3	NA	**8**	NA
4	NA	**9**	NA
5	10.95±1.00	**10**	6.61±0.50

Complex **7**, although being a macromolecule, showed dose dependent percentage inhibition of proliferation where its activity was significantly lesser than that of 5-FU as clear in the graph (Figure 2(A)). The proligand **5** and complex **10** show dose dependent responses similar to the standard drug (Figure 2(B)). However, the responses of complex **10** in

percentage inhibition of proliferation are comparable with those of the standard drug. In contrast, respective compound **5** shows markedly less antiproliferative activity as compared to the respective complex and 5-FU. The cell shrinkage and dead cells on HCT116 cell lines after treatment with complexes **7**, **10** and proligand **5** are depicted in Figure 2(C). Among the possible reasons, one of the possibilities is larger size of complex which might affect the permeability of molecule and rendering it inactive. Other reason might be the selective affinity of human colon cancer cell lines against the synthesized compounds as different cancer cell lines express different membrane receptors[45] which may be the reason of altered affinity.

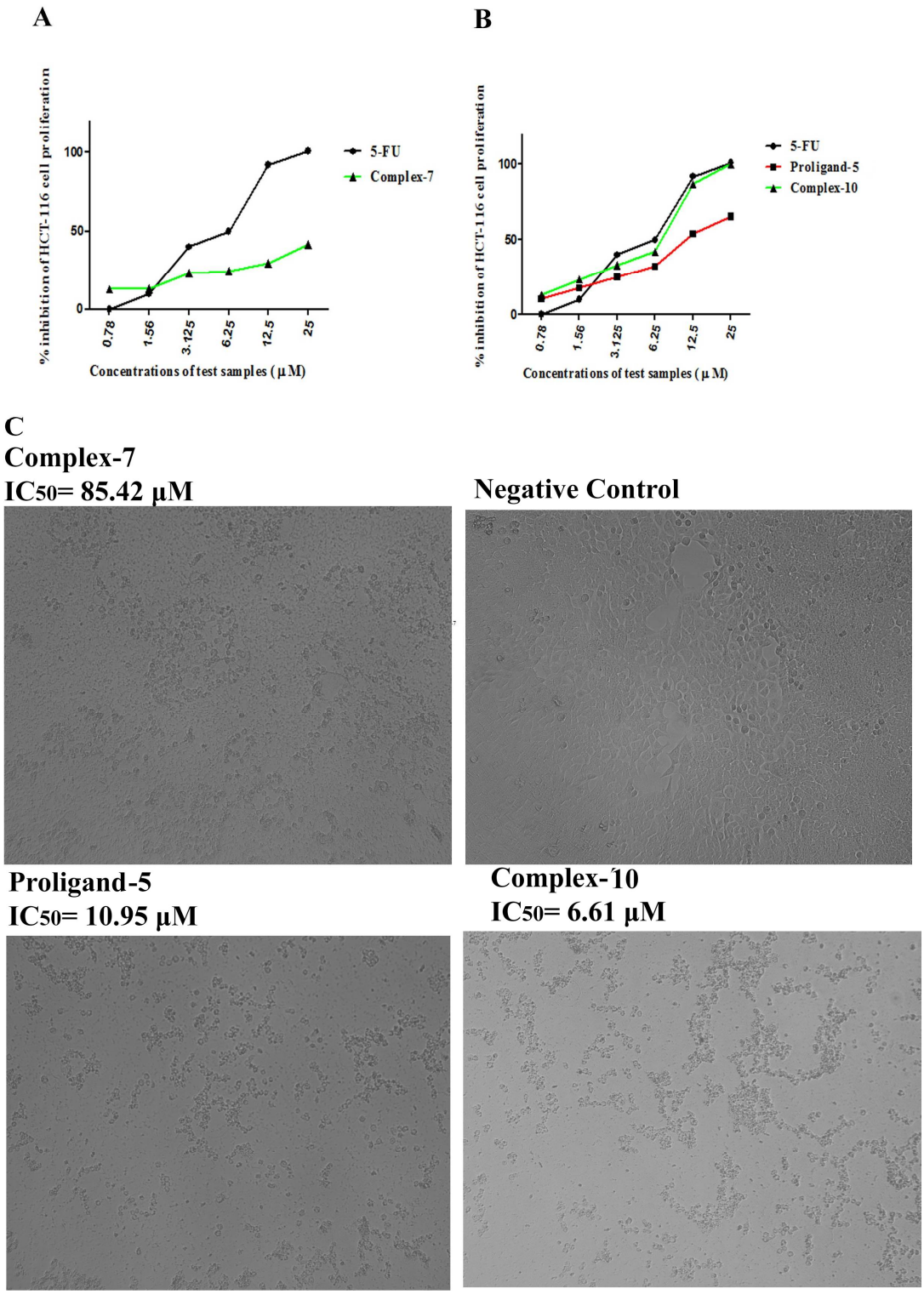


Figure 2: Graphs showing the effect of complex (A) **7** and (B) proligand **5** and complex **10** on percentage inhibition of HCT proliferation in comparison to standard drug 5- FU. (C) The altered morphology of human colorectal cell (cell images taken under a light microscope at ×200 magnifications with a digital camera).

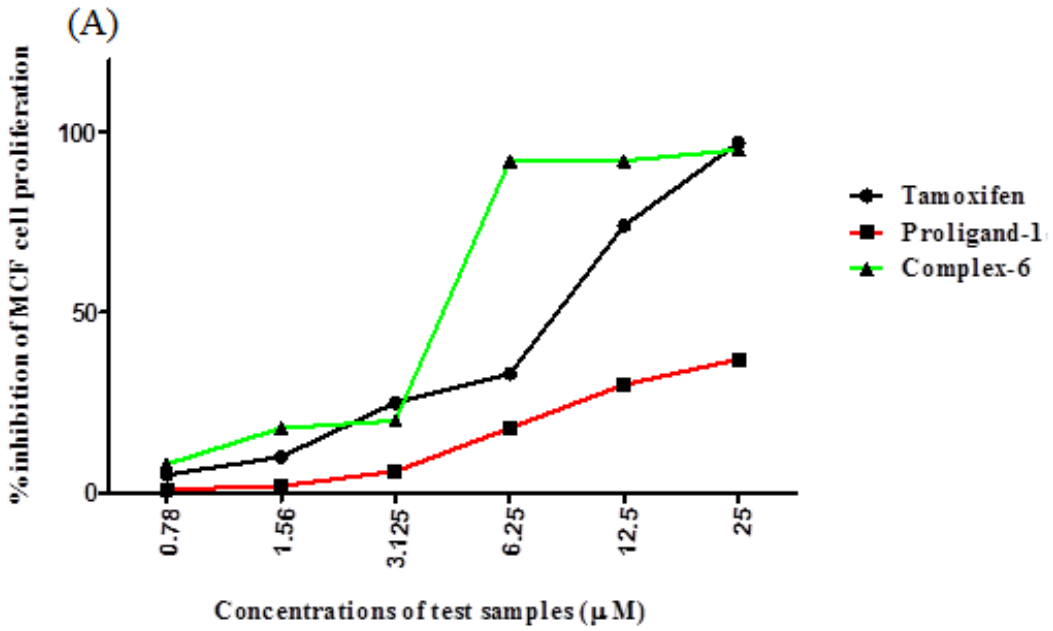
3.2. *In vitro* anticancer activities of tetra-NHC dinuclear silver(I) complexes and their respective proligands against the breast cancer (MCF-7) cell line

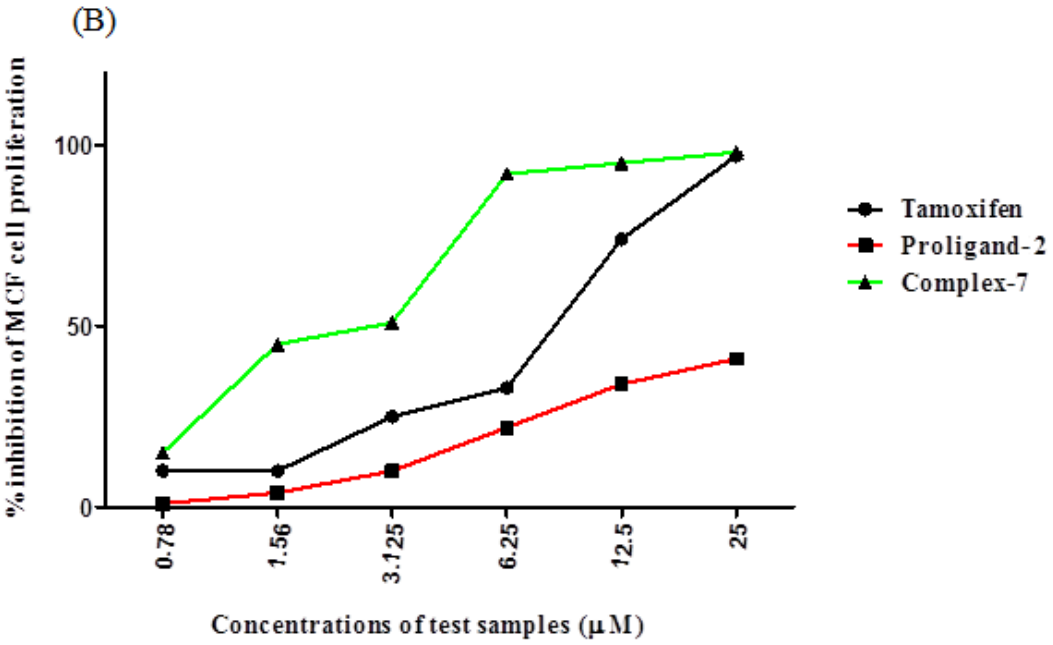
Unlike the activity displayed by the complexes of the tetra-NHC series on HCT 116, their responses are different on the breast cancer cell line. All proligands display lesser activity on the breast cancer cell lines (MCF-7) except the proligand **5** which shows slightly better activity. All complexes showed potent activity against MCF-7 with variable IC_{50} values as compared to Tamoxifen (Table 2).

Table 2: IC_{50} values of the proligands **(1-5)** and complexes (**6-10**) on the MCF-7 cell lines and the EA.hy926 cell lines using the standard Tamoxifen (IC_{50} = 7.5µM).

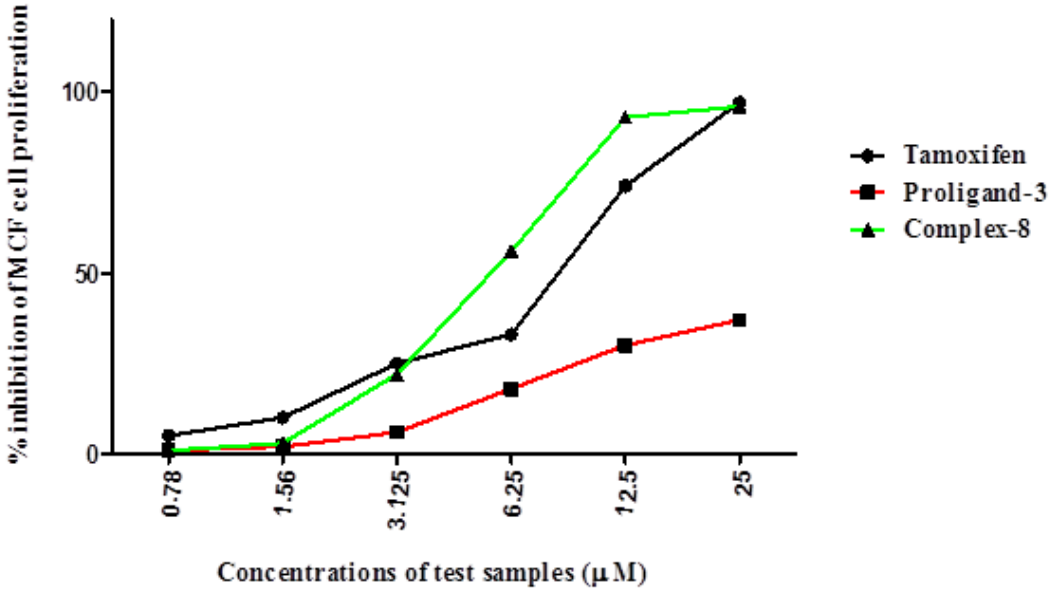
Proligands	IC_{50} (µM)		NHC-Ag(I) Complexes	IC_{50} (µM)	
	MCF-7	EA.hy		MCF-7	EA.hy
1	NA	NA	**6**	0.03±0.00	5.30±0.40
2	NA	NA	**7**	0.66±0.03	6.02±0.50
3	NA	NA	**8**	0.29±0.02	4.96±0.48
4	NA	NA	**9**	1.27±0.07	8.10±0.80
5	4.67±0.10	204.10±2.00	**10**	3.80±0.020	7.81±0.80

The tetra-NHC dinuclear silver(I) compounds not only show cytotoxicity against the breast cancer but also exhibit minimum toxicity on the normal cell lines signifying their cytotoxic effects on the breast cancer cell lines. Moreover, these complexes (**6-10**) displayed potent cytotoxicity as compared to their respective proligands (Figure 3).

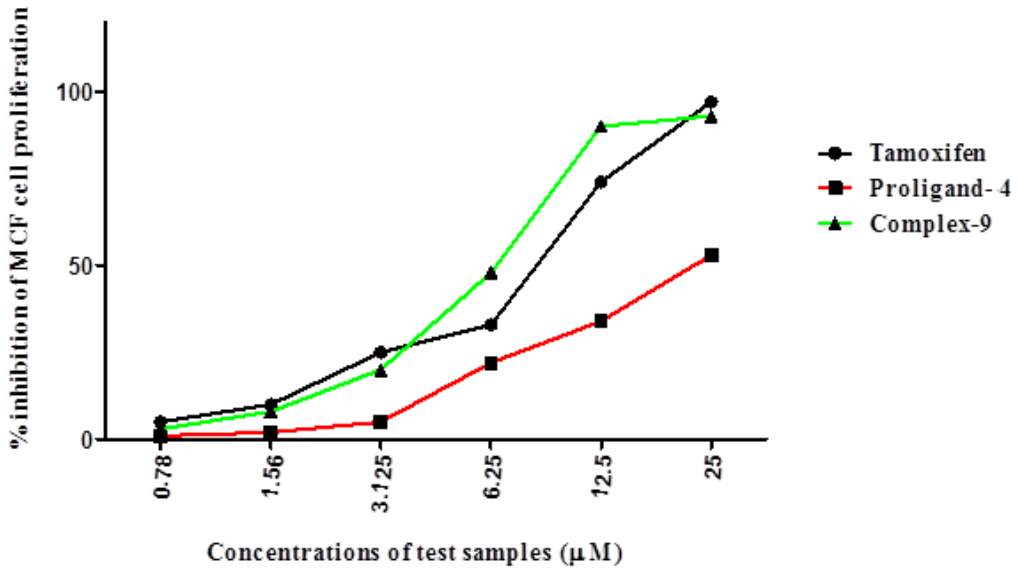

(A)
% inhibition of MCF cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µM)
Tamoxifen
Proligand-1
Complex-6


(B)
% inhibition of MCF cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µM)
Tamoxifen
Proligand-2
Complex-7

(C)


% inhibition of MCF cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µM)
Tamoxifen
Proligand-3
Complex-8

(D)


% inhibition of MCF cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µM)
Tamoxifen
Proligand- 4
Complex-9

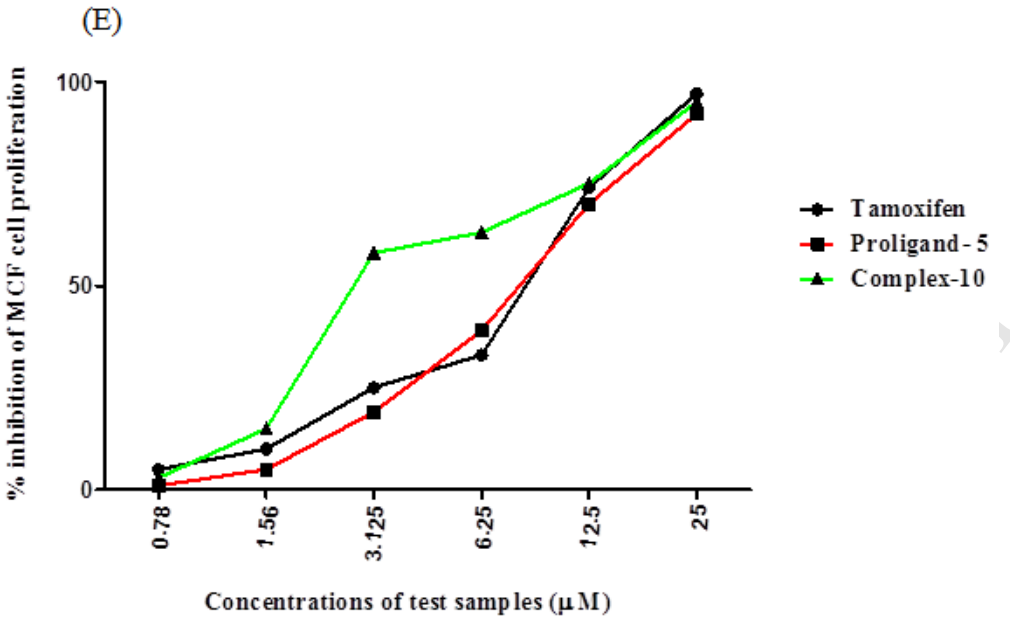


Figure 3: Dose dependent antiproliferative effects of (A) proligand **1** and complex **6**; (B) proligands **2** and complex **7**; (C) proligand **3** and complex **8**; (D) proligand **4** and complex **9**; (E) proligand **5** and complex **10** in comparison with the standard drug, Tamoxifen on the breast cancer cell lines.

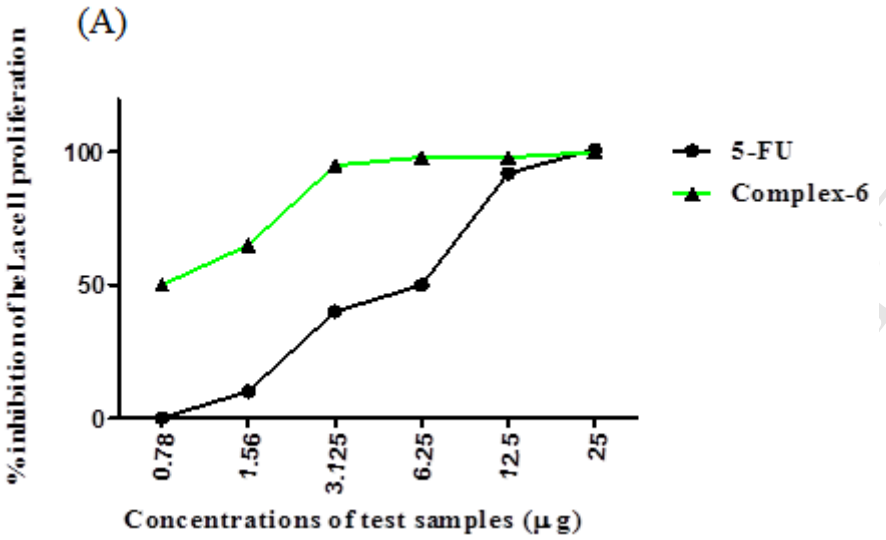
3.3. *In vitro* anticancer activities of the tetra-NHC dinuclear silver(I) complexes and their respective proligands against the human epitheloid cervix carcinoma (HeLa) cell line

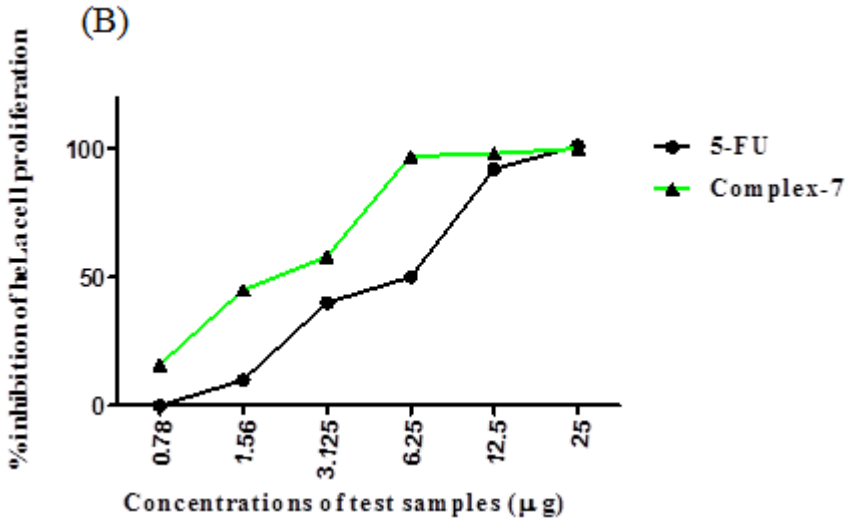
The imidazolium based mononuclear silver(I)-NHC complexes have been reported as poor anticancer agents against cervical cancer[31], thus we hypothesized that by increasing the number of NHC units and silver(I) centres may lead these tetra-NHC dinuclear silver(I) complexes active against cervical cancer cell line. All the synthesized proligands and complexes of tetra NHC-dinuclear silver(I) series were explored for their activity on HeLa cell lines by calculating the IC_{50} values. It was observed that all the compounds display similar fashion in IC_{50} values as it was in MCF-7 cell lines. All the complexes show activity on HeLa cell lines having variable IC_{50} values depending upon the terminal *N*-substitution (Table 3).

Table 3: IC_{50} values of proligand (**1**-**5**) and complexes (**6**-**10**) on the HeLa cell lines using 5-FU (10.2 µM) as the standard.

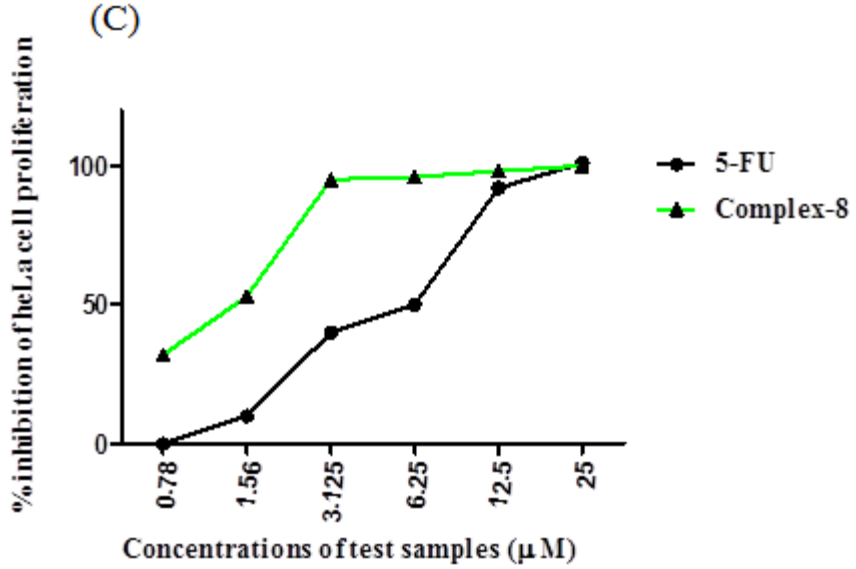
Proligands	IC_{50} (µM)	Ag(I)-NHC complexes	IC_{50} (µM)
1	NA	**6**	0.11±0.01
2	NA	**7**	2.08±0.10
3	NA	**8**	0.99±0.04
4	NA	**9**	2.60±0.20
5	5.97±0.44	**10**	1.10±0.20

These screened compounds were tested for their ability to inhibit proliferation of the cervical cancer cells at different doses in the range of 0.78-25 µM. The complexes **6**, **7** and **8** containing cyclopentyl, benzyl and *n*-butyl substitution respectively, on the tetra-NHC dinuclear silver(I) complexes, show similar dose dependent antiproliferation activities (Figure 4). The antiproliferation activities of these complexes are greater than those of 5-FU at lower and medium doses but are comparable at a maximum dose. The complexes **9** and **10** containing 2-methylene benzonitrile and *n*-decyl substitution, respectively on the tetra-NHC dinuclear silver(I) complexes, show similar dose dependent trends in antiproliferation activities that are comparable with the standard drug 5-FU. The proligand **5** containing *n*-decyl substitution also shows antiproliferative activity but lesser than the respective complex and 5-FU at all doses.

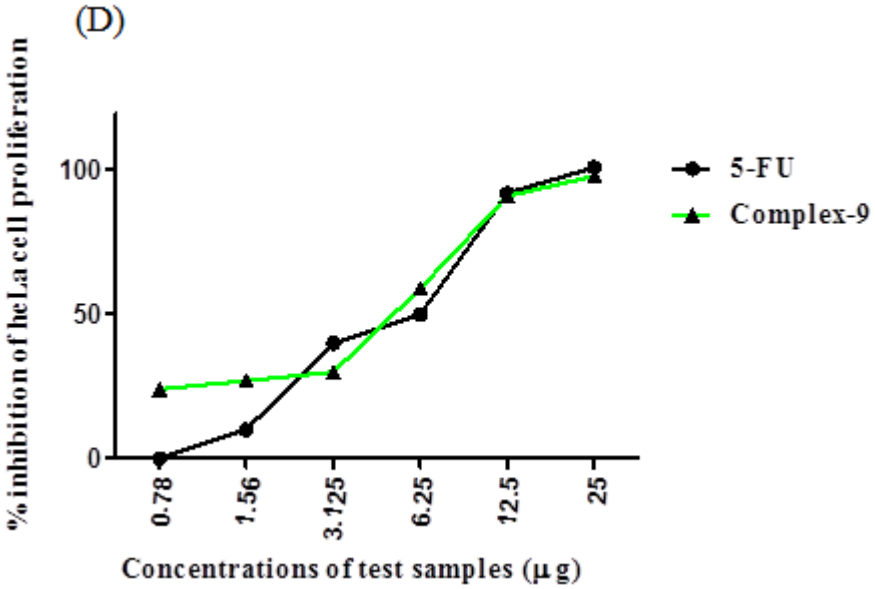

(A)
% inhibition of he La cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µg)
5-FU
Complex-6


(B)
% inhibition of he La cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µg)
5-FU
Complex-7

(C)


% inhibition of heLa cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µM)
5-FU
Complex-8

(D)


% inhibition of heLa cell proliferation
100
50
0
0.78
1.56
3.125
6.25
12.5
25
Concentrations of test samples (µg)
5-FU
Complex-9

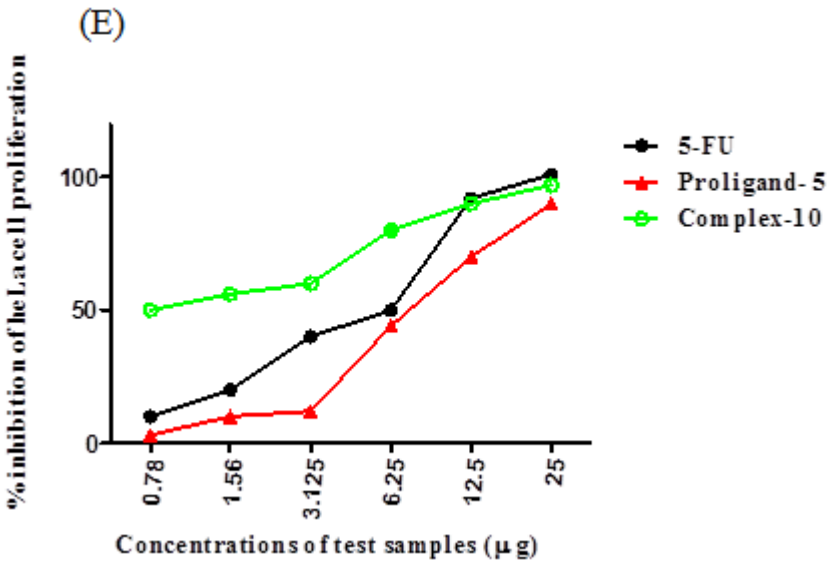


Figure 4: Dose dependent antiproliferative effects of (A) complex **6**; (B) complex **7**; (C) complex **8**; (D) complex **9**; (E) proligand **5** and the respective complex **10**, in comparison with the standard drug 5-FU against the human epitheloid cervix carcinoma (HeLa) cell lines.

It can be deduced from the anticancer study that modifying the substitution with cyclopentyl, benzyl, *n*-butyl, 2-methylenebenzonitrile and *n*-decyl groups on tetra-NHC dinuclear silver(I) complexes renders these compounds as potential agents against breast cancer and cervical cancer cell lines but selectively good anticancer agents for human colon cancer cell lines. Present study broadens the spectrum of tetra-NHC dinuclear silver(I) complexes by investigating their mode of cytotoxicity.

3.4. Study the mode of cytotoxicity of the selected tetra-NHC dinuclear silver(I) complex and its respective proligand

Cancer cells have an ability to proliferate and move to different organs of the body and that is the reason metastasis studies are considered as multistep studies including cell proliferation, migration and colony formation. Study of cancer cells migration in research is

of great interest as it is one of the attributions of metastatic progression. Cancer cells disseminate and spread throughout the body by migration and invasion through extracellular matrix, intravasate into the circulation and attached to distant organs sites and finally extravagate the distant foci[46]. Present study focusses on the role of tetra-NHC dinuclear silver(I) complexes as anticancer agent by inhibiting the cell migration and colony formation, cell culture wound closure, cell invasion and transwell migration that are widely employed in scientific community[47-48]. Complex **10** was selected to explore the anticancer action mechanism of the tetra-NHC dinuclear silver(I) complexes, while the respective proligand **5** was also recruited in this mechanistic study since among all complexes and proligands, only the complex **10** and proligand **5** show promising results in panel of cancer cell lines including HCT-116, MCF-7 and HeLa.

3.4.1 Cell migration assay for the proligand 5 and the complex 10

Antimigration of cancer cells have been employed in many studies as anticancer assay [49-50] . In this assay, two doses for proligand and the respective complex was administered in the cultured medium. The proligand **5** shows dose dependent responses which appeared maximum after 6 h of the experiment while the responses were reduced with time (Figure 5(A)). Similarly, the wound closure assay shows dose dependent responses as 6.25 μg/mL displayed 18.7 ± 4.23 % and 9.36 ± 0.83 % after 6 h and 12 h, respectively. At the same time, 12.5 μg/mL recorded highest value of wound closure as 32.46 ± 1.31 % and 16.24 ± 5.29 % after 6 h and 12 h respectively (Figure 5(B)).

This data indicates that the proligand **5** has an ability to suppress the cell motility in breast cancer cells while wound is totally closed after 12 hrs in negative control, so the antimetastatic activity of proligand **5** is by inhibiting cell migration in breast cancer cell line. Inhibition of metastasis of cancer cells in breast cancer cell lines have been studied

previously for anticancer agent[51]. The wound healing ability of the proligand **5** confirms the selectivity of this compound against the cancer cell lines and shows its potential as an anticancer agent by antimigration assay.

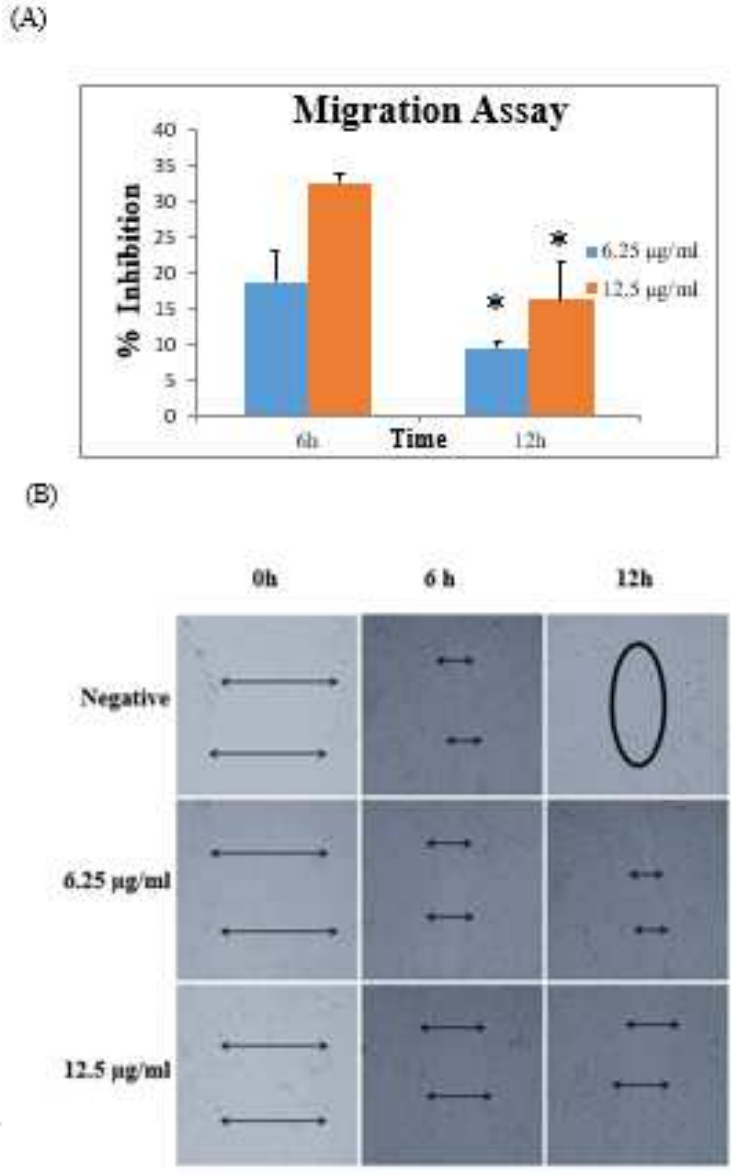


Figure 5: (**A**) Antimigration of the cancer cells data for proligand **5** after 6 h and 12 h of administration of doses. (**B**) Images of the breast cancer cell line (MCF-7) in the cell migration assay. The cells were grown in the 6-well cell culture plates and a wound was created in the center of each well when the wells were confluent. These were then treated with the proligand **5** in two concentrations of 6.25 and 12.5 µg/mL and the images of the cells were taken under an inverted phase-contrast microscope at ×4 magnification at 0 h (zero), 6 h and 12 h after the treatment with the proligand **5**. The straight lines in the figure

indicate the cell-free zone while the circles indicate the sites where the cell migration filled the free zone.

Similarly, the complex **10** displays highest activity in the wound closure by inhibiting the migration of the breast cancer cells after 6 h, these responses are reduced with the passage of time which indicates that the complex has achieves the peak plasma concentration after 6 h and displays maximum activity (Figure 6(A)). The antimigration assay shows that 2.5 μg/mL displays 15.37 ± 3.43 % and 5.54 ± 1.07 % after 6 h and 12 h respectively, while the 5 μg/mL records the highest value of wound closure as 32.9 ± 5.62 % and 6.24 ± 3.76 % after 6 h and 12 h respectively (Figure 6(B)). It is important to mention here that with complex **10**, the lowest administered doses are 2.5 and 5 μg/mL, whereas for the proligand **5**, the lowest doses are 6.25 and 12.5 μg/mL. Similar to that of proligand **5**, this wound healing ability of complex **10** confirms its selectivity against the cancer cell lines rather than normal cell lines thus further strengthen the confidence of selective cytotoxicity. Inhibition of cell migration in HCT cancer cell lines by dinuclear silver(I)-NHC were studied before but present study introduced macromolecule of tetra-NHC dinuclear silver(I) complex that stands with the novel findings as antimetastatic role of tetra-NHC dinuclear silver(I) complex in breast cancer cell line by inhibiting the cell migration which have never been explored before.

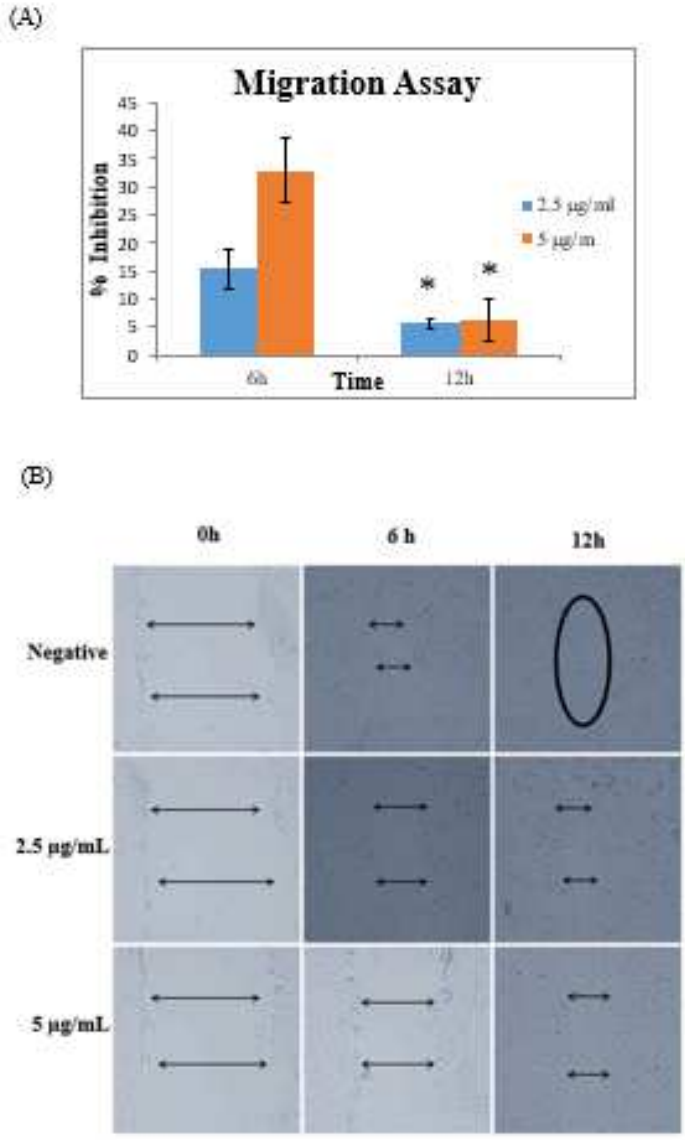


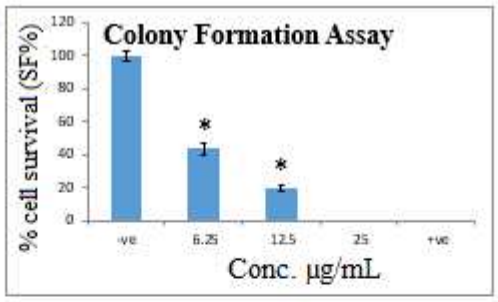
Figure 6: (**A**) Antimigration of the cancer cells data for complex **10** after 6h and 12 h of administration of doses. (**B**) Images of the breast cancer cell line (MCF-7) in the cell migration assay. The cells were grown in 6-well cell culture plates and a wound was created in the center of each well after the wells were confluent. The cells were then treated with complex **10** in two concentrations of 2.5 µg/mL and 5 µg/mL and the images of the cells were taken under an inverted phase-contrast microscope at ×4 magnification at 0 h (zero), 6 h and after 12 h of treatment with the complex **10**. The straight lines indicate the cell-free zone while the circles indicate the sites where the cell migration has filled the free zone.

3.4.2. Colony formation assay for the proligand 5 and the complex 10

To evaluate further, inhibition of colony formation of cancer cells have been employed in present study which is previously reported in many studies to find anticancer mechanism[50,52]. An ideal anticancer agent should inhibits the colony formation and be selectively toxic to the cancer cells. The studied data is presented as percentage of cell survival (Figure 7(A)). Data depicted the trend that lesser the percentage survival, the greater is the anticancer activity as negative control has 100% survival while positive control showed 0% cell survival.

The administrations of doses are in the range of 6.25-25 μg/mL show dose dependent responses on the survival of the cancer cells. Higher the concentration of proligand **5**, the lower was the percentage survival of the breast cancer cells and greater the anticancer activity as clear in Figures 7 (A and B). It can be deduced from the present assay that the proligand **5** shows an anticancer potential by inhibiting the colony formation, rendering toxicity on the cancer cells of breast cancer cell lines thereby reducing the cell survival and the highest dose of 25 μg/mL shows responses comparable to the reference control, Tamoxifen.

(A)



Conc. µg/mL	Negative control	6.25	12.5	25	Positive control
SF%	100±3.48	43.64±3.75	19.54±1.87	0	0

(B)

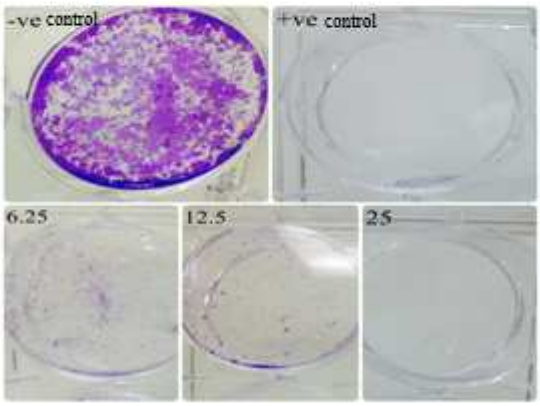
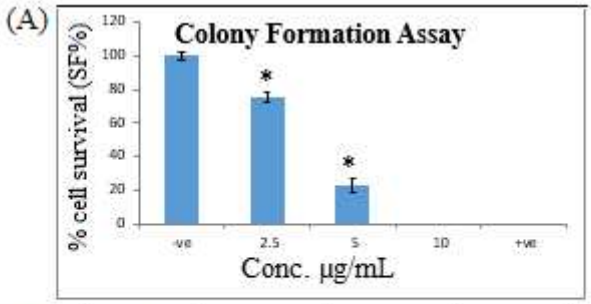


Figure 7: (A) Data showing the percentage of cell survival after administration of different doses 6.25-25 µg/mL of proligand **5** on the breast cancer cell line. (B) Photographs of the cell culture plates showing the cell survival after administration of different doses 6.25-25 µg/mL of proligand **5** on the breast cancer cell line.

Complex **10** shows maximum activity at lower doses when compared to doses of proligands in the cell migration assay, therefore the same concentrations 2.5, 5 and 10 µg/mL of this complex were investigated in the colony formation assay. It was noticed that increasing the dose of complex **10** reduces the percentage of cell survival of the breast cancer cell line, maximum response is observed at highest dose which is 10 µg/mL and these responses are comparable to the standard drug used in the present assay (Figure 8(A)). The cell survival is evident in the photograph of culture plates seeded with breast cancer cell (Figure 8(B)). It is noteworthy to mention that complex **10** shows maximum responses at

doses lower than the doses used for proligand **5** which can be attributed to the presence of two silver(I) ions in the complex.

The study of mode of cytotoxicity reveals that the proligand **5** and the complex **10** show anticancer potential by inhibiting the cell migration and colony formation of the breast cancer cells. It can be deduced from our investigation that the complex **10** requires less dose to produce therapeutic responses on the breast cancer cell lines as compared to the respective proligand **5** and this feature can be attributed to the anticancer potential of silver(I).



Conc. µg/mL	Negative control	2.5	5	10	Positive control
SF%	100±2.45	75.43±2.8	22.58±3.86	0	0

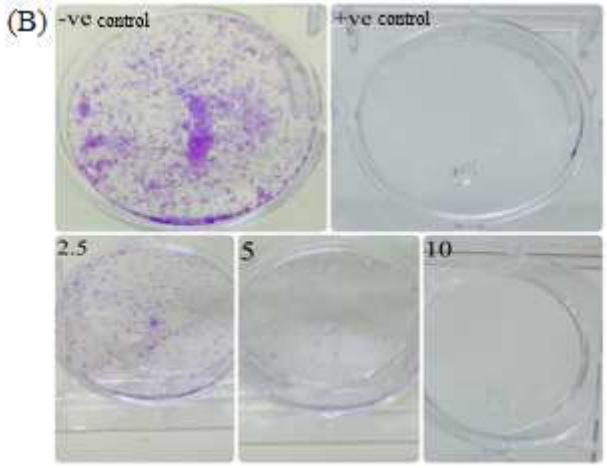


Figure 8: (A) Data showing the percentage of cell surviving after administration of different doses 2.5-10 µg/mL of complex **10** on breast cancer cell line. (B) Photographs of the cell culture plates showing the cell survival after administration of different doses 2.5-10 µg/mL of complex **10** on the breast cancer cell line.

4. Conclusion

In this study five tetra-NHC dinuclear silver(I) complexes, **6-10** have been successfully derived from the five new tetrakis benzimidazolium salts synthesized by the newly introduced synthetic scheme. All the proligands and the respective tetra-NHC dinuclear silver(I) complexes have been tested for anticancer potential against HCT-116, MCF-7 and HeLa cell lines. The proligand with *n*-decyl terminal substitution was the only active proligand against the panel of selected cancer cell lines. All the complexes show better activity on MCF-7 and HeLa cell lines as compared to the HCT-116. The selected tetra-NHC compounds (**5** and **10**) show anticancer potential by inhibiting the cell migration and colony formation ability of cancer cells on MCF-7.

5. Acknowledgement

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6. References

1. H. W. Wanzlick, H. J. Schonherr, Angew. Chem. Int. Ed., 7 (1968)**,** 141-142.
2. K. Öfele, J. Organomet. Chem., 12 (1968) P42-P43.
3. A. J. Arduengo III, R. L. Harlow, M. Kline, J. Am. Chem Soc., 113 (1991) 361-363.
4. D. Bourissou, O. Guerret, F. P. Gabbai, G. Bertrand, Chem. Rev. 100 (2000) 39-92.
5. R. Corberán, E. Mas-Marzá, E. Peris, Eur. J. Inorg. Chem., 2009 (2009) 1700-1716.
6. M. Poyatos, J. A. Mata, E. Peris, Chem. Rev., 109 (2009) 3677-3707.
7. X. Hu, Y. Tang, P. Gantzel, K. Meyer, Organometallics, 22 (2003) 612-614.
8. A. Rit, T. Pape, F. E. Hahn, J. Am. Chem. Soc., 132 (2010) 4572-4573.

9. F. E. Hahn, V. Langenhahn, T. Lugger, T. Pape, D. Le Van, Angew. Chem. Int. Ed. 44 (2005) 3759-3763.

10. R. McKie, J. A. Murphy, S. R. Park, M. D. Spicer, S. Z. Zhou, Angew. Chem. Int. Ed. 46 (2007) 6525-6528.

11. F. E. Hahn, C. Radloff, T. Pape, A. Hepp, Chem. Eur. J., 14 (2008) 10900-10904.

12. T. Pape, F. E. Hahn, Dalton Trans. 42 (2013) 7330-7337.

13. P. J. Altmann, D. T. Weiss, C. Jandl, F.E. Kuhn, Chem. Asian J., 11 (2016) 1597-1605.

14. E. K. Bullough, M. A. Little, C. E. Willans, Organometallics 32 (2013) 570-577.

15. B. N. Ahamed, R. Dutta, P. Ghosh, Inorg. Chem., 52 (2013) 4269-4276.

16. Z. Lu, S. A. Cramer, D. M. Jenkins, Chem.Sci., 3 (2012) 3081-3087.

17. D. T. Weiss, S. Haslinger, C. Jandl, A. Pothig, M. Cokoja, F. E. Kuhn, Inorg. Chem., 54 (2015) 415-417.

18. D. T. Weiss, P. J. Altmann, S. Haslinger, C. Jandl, A. Pothig, M. Cokoja, F. E. Kuhn, Dalton Trans., 44 (2015) 18329-18339.

19. C. Segarra, G. Guisado-Barrios, F. E. Hahn, E. Peris, Organometallics, 33 (2014) 5077-5080.

20. T. Fatima, R. A. Haque, M. A. Iqbal, M. R. Razali, J. Organomet. Chem., 831 (2017) 50-54.

21. T. Fahlbusch, M. Frank, G. Maas, J. R. Schatz, Organometallics, 28 (2009) 6183-6193.

22. R. Wang, B. Twamley, J. N. M. Shreeve, J. Org. Chem., 71 (2006) 426-429.

23. J. A. Mata, A. R. Chianese, J. R. Miecznikowski, M. Poyatos, E. Peris, J. W. Faller, R. H. Crabtree, Organometallics, 23 (2004) 1253-1263.

24. D. Gnanamgari, E. L. O. Sauer, N. D. Schley, C. Butler, C. D. Incarvito, R. H. Crabtree, Organometallics, 28 (2008) 321-325.

25. J. Ramirez, R. Corberan, M. Sanau, E. Peris, E. Fernandez, Chem. Commun., (2005) 3056-3058.

26. R. Corberan, J. Ramirez, M. Poyatos, E. Peris, E. Fernandez, Tetrahedron: Asymmetry, 17 (2006) 1759-1762.

27. A. Piermattei, S. Karthikeyan, R. P. Sijbesma, Nature Chem., 1 (2009) 133-137.

28. A. Kascatan-Nebioglu, M. J. Panzner, C. A. Tessier, C. L. Cannon, W. J. Youngs, Coord. Chem. Rev., 251 (2007) 884-895.

29. S. Ray, R. Mohan, J. K. Singh, M. K. Samantaray, M. M. Shaikh, D. Panda, P. Ghosh, J. Am. Chem. Soc., 129 (2007) 15042-15053.

30. R. A. Haque, P.O. Asekunowo, M. R. Razali, J. Coord. Chem., 67 (2016) 2131-2147.

31. A. M. Doug, M. H. Khadijah, J. P. Matthew, J. D. Andrew, H. Y. Yang, J. Y. Wiley, Metal-Based Drugs (2008)**,** 2008.

32. T. J. Siciliano, M. C. Deblock, K. M. Hindi, S. Durmus, M. J. Panzner, C. A. Tessier, W. J. Youngs, J. Organomet. Chem., 696 (2011) 1066-1071.

33. M.-L. Teyssot, A.-S. Jarrousse, M. I. Manin, A. I. Chevry, S. P. Roche, F. D. R. Norre, C. Beaudoin, L. Morel, D. Boyer, R. Mahiou, Dalton Trans., (2009) 6894-6902.

34. S. Patil, A. Deally, B. Gleeson, H. Muller-Bunz, F. Paradisi, M. Tacke, Metallomics, 3 (2011) 74-88.

35. I. Fichtner, J. Cinati, M. Michaelis, L. C. Sanders, R. Hilger, B. N. Kennedy, A. L. Reynolds, F. Hackenberg, G. Lally, S. J. Quinn, I. McRae, M. Tacke, Letters in Drug Design & Discovery, 9 (2012) 815-822.

36. D. C. F. Monteiro, R. M. Phillips, B. D. Crossley, J. Fielden, C. E. Willans, Dalton Trans., 41 (2012) 3720-3725.

37. T. Fatima, R. A. Haque, M. R. Razali, A. Ahmad, M. Asif, K. Ahamed, B. Mohamed, A. Abdul Majid, Appl. Organometal. Chem., 31 (2017) 3735.

38. A. F. Aisha, K. M. Abu-Salah, Z. Ismail, A. M. S. A. Majid, BMC Complement. Altern. Med., 12 (2012) 104.

39. R. B. Badisa, S. F. Darling-Reed, P. Joseph, J. S. Cooperwood, L. M. Latinwo, C. B. Goodman, Anticancer Res., 29 (2009) 2993-2996.

40. P. F. de Oliveira, J. M. Alves, J. L. Damasceno, R. A. M. Oliveira, H. J. Dias, A. E. M. Crotti, D. C. Tavares, Revista Brasileira de Farmacognosia, 25 (2015) 183-188.

41. M. Asif, M. A. Iqbal, M. A. Hussein, C. E. Oon, R. A. Haque, M. B. K. Ahamed, A. S. A. Majid, A. M. S. A. Majid, Eur. J. Med. Chem., 108 (2016) 177-187.

42. C.-C. Liang, A. Y. Park, J.-L Guan, Nat. Protoc., 2 (2007) 329-333.

43. N. A. Franken, H. M. Rodermond, J. Stap, J. Haveman, C. Van Bree, Nat. Protoc., 1 (2006) 2315-2319.

44. W. Liu, R. Gust, Chem. Soc. Rev., 42 (2013) 755-773.

45. W.-C. Huang, F.-M. Lin, T.-H. Chang, K.-W. Liao, H.-D. Huang, Int. J. Bioinform Res Appl., 8 (2012) 192-209.

46. P. S. Steeg, Nat. Med., 12 (2006) 895-904.

47. R. D. Castellone, N. R. Leffler, L. Dong, L. V. Yang, Cancer Lett., 312 (2011) 197-208.

48. T. Albini, Pathol Onco Res., 4 (1998) 230-241.

49. A. Klimaszewska-Wiśniewska, M. Hałas-Wiśniewska, M. Izdebska, M. Gagat, A. Grzanka, D. Grzanka, Acta Histochem., 119 (2017) 99-112.

50. S. S. Dahham, L. E. A. Hassan, M. B. K. Ahamed, A. S. A. Majid, A. M. S. A. Majid, N. N. Zulkepli, BMC Complement Altern Med., 16 (2016) 236.

51. L. G. Naso, I. Badiola, J. M. Clavijo, M. Valcarcel, C. Salado, E. G. Ferrer, P. A. Williams, Bioorganic Med. Chem., 24 (2016) 6004-6011.

52. M. A. Iqbal, M. I. Umar, R. A. Haque, M. B. K. Ahamed, M. Z. B. Asmawi, A. M. S. A. Majid, J. Inorg. Biochem., 146 (2015) 1-13.

Highlights

- Open chain tetrakis benzimidazolium salt with a new architecture that serves as a precursor for the tetra NHC ligand.
- The increased activity of Ag(I)-NHCs have been attributed to their increased stability.
- By increasing the number of NHC units and silver(I) centres, these may lead the tetra-NHC dinuclear silver(I) complexes active against cervical cancer cell line.