Journal of Molecular Structure 1222 (2020) 128890

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstr

Tri *N*-Heterocyclic Carbene Trinuclear Silver(I) complexes: Synthesis and *In Vitro* cytotoxicity studies

Tabinda Fatima[a,d], Rosenani A. Haque[a], Ashfaq Ahmad[b], Loiy Elsir Ahmed Hassan[c], Mohamed B Khadeer Ahamed[c], AMS Abdul Majid[c], Mohd.R. Razali[a,*]

[a] School of Chemical Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia.
[b] Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, 23298-0613, USA.
[c] EMAN Research and Testing Laboratory, School of Pharmacy, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia.
[d] Department of Chemistry, COMSATS University Islamabad, Islamabad Campus, 45550, Pakistan.

ARTICLE INFO

Article history:
Received 18 February 2020
Revised 10 July 2020
Accepted 11 July 2020
Available online 12 July 20202

Keywords:
NHC ligands
Tris benzimidazolium salts
Antiproliferative activity
Trinuclear silver(I)

ABSTRACT

Synthesis of ethylene linked tris benzimidazolium bromide salts that serve as precursors for the tri-NHC ligands in the respective tri-NHC trinuclear silver(I) complexes is described (NHC = *N*-heterocyclic carbene). Different wingtip substituents were selected (benzyl, *n*-butyl, cyclopentyl, 2-methylene benzonitrile, *n*-decyl) to furnish five new tris benzimidazolium salts (**1**-**5**) and the respective tri-NHC trinuclear silver(I) complexes (**6**-**10**). The synthesized and characterized compounds (**1**-**10**) were evaluated for their cytotoxic potential on human colon, human breast and human epitheloid cervix cancer cell lines by determining their IC_{50} values and evaluating their antiproliferative activity against the selected cell lines. The tested compounds were found to be good anticancer agents against breast cancer and cervical cancer cell lines and selectively good anticancer agents for the human colon cancer cell line.

1. Introduction

The *N*-heterocyclic carbenes are well recognized class of ligands in organometallic chemistry due to their ability to form stable complexes with low and high oxidation state metals [1–3]. This class of ligands offer the advantages of structural diversity and various coordination modes over other known ligands. In addition to the widespread research on the synthesis and applications of mono and bis *N*-heterocyclic carbenes, the introduction of tris and tetrakis NHC complexes has further opened up a new avenue and so far there is still a very few reports addressing these poly NHC systems.

The literature known tris azolium salts have been reported to be synthesized in a single step reaction, by reacting an azole with a tripodal precursor [4–8]. A multistep scheme for the synthesis of tris-NHC ligands has been reported recently [9]. A new designed multistep scheme for the synthesis of tris benzimidazolium salts was introduced by our group. Our designed scheme was different from the previous and the recent reported tri NHC proligands as it involved the reaction of unsubstituted benzimidazole with the synthesized 3-(2-bromoethyl)-1-substituted benzimidazolium bromide [10]. After introducing a new strategy towards the synthesis of tris azolium salts (Fig. 1) [10] we put forth our efforts to synthesize tris benzimidazolium salts having different wingtip substituents (benzyl, *n*-butyl, cyclopentyl, 2-methylene benzonitrile, *n*-decyl). The resulting salts serve as precursors for the tri-NHC ligands which were generated by their complexation with silver(I) ions.

The tri-carbene metal complexes reported so far have been studied for catalysis [7,8,11,12], luminescence [9] and transmetallation [13] properties. However, the biological studies on such complexes still remain elusive in contrast to the anticancer and antibacterial properties studied for the mono and di-NHC metal complexes [14-18]. The Ag(I)-NHC complexes, compared to the earlier known ionic silver drugs like silver sulfazide and silver nitrate, were found to have better efficacy as they involve slow and sustained release of silver ions [19]. Taking advantage of this capability of Ag(I)-NHC complexes, apart from the potent antibacterial properties the anticancer perspective of mono- and di- Ag(I)-NHC complexes have also been investigated in several studies. The studied anticancer mechanism of action of these complexes showed that they are not genotoxic as no modifications in the cell cycle have so far been observed [20,21]. Further investigations by using fluorescent Ag(I)-NHC complexes confirmed that these complexes target mitochondria and offers apoptotic cell death [22]. Among the poly NHC Ag(I) complexes the Ag(I)-tetra NHC complexes were investigated for antiproliferative activity. These Ag(I)

* Corresponding author.
E-mail address: mohd.rizal@usm.my (Mohd.R. Razali).

https://doi.org/10.1016/j.molstruc.2020.128890
0022-2860/

complexes were found to be anticancer agents by inhibiting the cell migration and colony formation of cancer cells [23].

As we have studied and reported the cytotoxicity of mono-, di- and tetra-NHC silver(I) complexes against various cancer cell lines [24–26], thus in keeping the continuation of our studies, the tri-NHC silver(I) complexes were prepared and evaluated for their anticancer potential against various cancer cell lines. The present study aimed to probe whether the tri-NHC metal complexes having three Ag(I) ions per complex, furnishes greater number of silver(I) ions with a more sustained release in the cancer cells as compared to mono-NHC Ag(I) complexes having one, di- and tetra-NHC Ag(I) complexes having two silver centres per complex. Further in order to get insight into the effect of substituents on the delivery of the tri-NHC Ag(I) complexes into the cancer cells different substituents (benzyl/ n-butyl/ cyclopentyl/ 2-methylene benzonitrile/ n-decyl) have been selected.

2. Experimental

2.1. Materials and instruments

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

2.2. Synthesis

The precursor salts 3-(2-bromoethyl)-1-(benzyl/ n-butyl/ cyclopentyl/ 2-methylene benzonitrile/ n-decyl) benzimidazolium bromides (**I-V**), proligand **1** (Benzyl substituted tris benzimidazolium bromide salt) and respective silver(I) complex **6** were prepared according to the reported procedure [27].

Synthesis of tris benzimidazolium bromides (2-5)

2.2.1. n-Butyl substituted tris benzimidazolium bromide (2)

Compound **II** (1.30 g, 3.60 mmol) and benzimidazole (0.21 g, 1.8 mmol) were dissolved in a mixture of 1,4-dioxane (20 mL) and methanol (2 mL). The reaction mixture was heated under reflux for 32 hours. The tris benzimidazolium bromide salt appeared as white precipitate, which was filtered and washed with 1,4-dioxane and dichloromethane sequentially. The product obtained was dried in fume cupboard. Yield: 0.71 g (51%). Anal. Calc. for $C_{33}H_{41}Br_3N_6$: C, 52.03; H, 5.38; N, 11.03. Found: C, 52.43; H, 5.42; N, 10.80. FTIR (KBr) υ (cm^{-1}): 3166, 3105 (C_{arom}-H), 2967, 2877 (C_{aliph}-H), 1614, 1489, 1469 (C_{arom}=C_{arom}), 1568 (C_{arom}-N), 1282 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.92 (6H, 2 × CH_3, t, J = 7.5 Hz), 1.29 (4H, 2 × CH_3-CH_2-, m), 1.84 (4H, 2 × CH_2-CH_2-CH_2, m), 4.45 (4H, 2 × -CH_2-N, t, J = 7.0 Hz,), 5.28 (8H, 2 × N-CH_2-CH_2-N, s), 7.47-8.04 (12H, Ar-H, m), 9.91 (2H, NCHN, s), 10.20 (1H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 13.0 (CH_3); 18.9, 30.2, 45.5, 47.0 (CH_2); 112.6, 113.6, 127.2, 127.8, 130.8, 131.2 (Ar-C); 143.2, 144.4 (NCHN).

2.2.2. Cyclopentyl substituted tris benzimidazolium bromide (3)

Salt **3** was prepared following the same procedures as described for salt **2** except that salt **II** was replaced by **III** (1.05 g, 2.60 mmol), benzimidazole (0.16 g, 1.30 mmol). Salt **3** was collected as white precipitate. Yield: 0.57 g (57%). M.p.: 286-290°C. Anal. Calc. for $C_{35}H_{41}Br_3N_6$: C, 53.50; H, 5.09; N, 10.70. Found: C, 53.77; H, 5.23; N, 10.88. FTIR (KBr) υ (cm^{-1}): 3163, 3102 (C_{arom}-H); 2968, 2884 (C_{aliph}-H), 1618, 1488, 1431 (C_{arom}=C_{arom}), 1561 (C_{arom}-N), 1275 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 1.73-2.27 (16H, 8 × CH_2 of cyclopentyl, m), 4.99 (2H, -CH-, m), 5.23 (8H, 2 × N-CH_2-CH_2-N, s), 7.42-8.13 (12H, Ar-H, m), 10.12 (2H, NCHN, s), 10.17 (1H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 22.9, 29.1, 31.3, 45.6, 59.6 (CH_2); 112.5, 114.1, 127.4, 127.8, 130.6, 131.2 (Ar-C); 143.1, 144.5 (NCHN).

2.2.3. 2-Methylenebenzonitrile substituted tris benzimidazolium bromide (4)

Salt **4** was prepared following the same procedures as described for salt **2** except that salt **II** was replaced by **IV** (0.73 g, 1.62 mmol), benzimidazole (0.12 g, 0.83 mmol). Salt **4** was collected as white precipitate. Yield: 0.40 g (57%). M.p.: 275-279°C. Anal. Calc. for $C_{41}H_{35}Br_3N_8$: C, 55.97; H, 3.98; N, 12.74. Found: C, 56.31; H, 4.04; N, 12.47. FTIR (KBr) υ (cm^{-1}): 3159 (C_{arom}-H), 2968 (C_{aliph}-H), 2228(C≡N), 1616, 1491, 1446 (C_{arom}=C_{arom}), 1568 (C_{arom}-N), 1287 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 5.02 (8H, 2 × -CH_2-CH_2-, s), 5.80 (4H, 2 × CH_2-Ar, m), 7.46-8.20 (20H, H-Ar, m), 10.01 (2H, NCHN, s), 10.30 (1H, NCHN, s).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 45.6, 46.2, 49.2 (CH_2); 111.7, 112.9, 113.1, 113.8, 116.5, 127.6, 127.8, 129.4, 129.9, 130.8, 131.2, 133.7, 134.8 (Ar-C); 143.1, 144.3 (NCHN).

2.2.4. n-decyl substituted tris benzimidazolium bromide (5)

Salt **5** was prepared following the same procedures as described for salt **2** except that salt **II** was replaced by **V** (1.04 g, 2.50 mmol), benzimidazole (0.15 g, 1.21 mmol). Salt **5** was collected as white precipitate. Yield: 0.60 g (60%). Anal. Calc. for $C_{45}H_{65}Br_3ClN_6$: C, 61.05; H, 7.34; N, 9.49. Found: C, 61.38; H, 6.98; N, 9.63. FTIR (KBr) υ (cm^{-1}): 3166, 3109 (C_{arom}-H); 2926, 2855 (C_{aliph}-H), 1614, 1458 (C_{arom}=C_{arom}), 1572 (C_{arom}-N), 1271 (C_{aliph}-N). ^{1}H NMR (500 MHz, DMSO-d_6) δppm: 0.85 (6H, 2 × CH_3, t, J = 7.5 Hz), 1.22-1.25 (28H, 14 × -CH_2-, m), 1.77-1.79 (4H, 2 × -CH_2-, m), 4.44 (4H, 2 × -CH_2-N, t, J = 7.0 Hz), 5.22 (8H, 2 × -CH_2-CH_2-, s), 7.46-7.80 (8H, Ar-H, m), 8.09-8.20 (4H, Ar-H, m), 10.16 (2H, NCHN, s), 10.37 (1H, NCHN, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 13.8(CH_3); 22.1, 25.6, 28.4, 28.6, 28.7, 28.9, 31.2, 45.5, 46.6 (CH_2); 111.2, 113.4, 113.7, 126.5, 130.7, 131.4(Ar-C); 142.8, 144.2 (NCHN).

Synthesis of tri-NHC trinuclear Ag(I) complexes (7-10)

2.2.5. n-Butyl substituted tri NHC-Ag(I) complex (7)

Salt **2** (0.25 g, 0.35 mmol) was dissolved in methanol (50 mL) and silver oxide (0.23 g, 1.00 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. 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 obtained silver complex is in bromide form so it was subjected to metathesis using potassium hexafluorophosphate (0.18 g, 1.00 mmol). The mixture was stirred for 2 hours and the resulting precipitates were filtered, washed with distilled water and left to dry at room temperature. The silver complex **7** was collected as white precipitate. Yield: 0.27 g (43%). M.p.: 254-257°C. Anal. Calc. for $C_{66}H_{76}$ $Ag_3F_{18}N_{12}P_3.H_2O$: C, 43.69; H, 4.30; N, 9.26. Found: C, 43.30; H, 3.85; N, 9.19. FTIR (KBr) υ (cm^{-1}): 3063 (C_{arom}-H), 2958, 2873 (C_{aliph}-H), 1614, 1481, 1450 (C_{arom}=C_{arom}), 1271 (C_{aliph}-N). ^{13}C NMR (125 MHz, acetonitrile-d_3) δ ppm: 12.6 (CH_3); 19.1, 31.8, 43.8, 49.5, 50.1 (CH_2); 108.9, 110.6, 116.0, 121.2, 124.8, 133.7 (Ar-C); 187.8 (C-Ag, br d).

2.2.6. Cyclopentyl substituted tri NHC-Ag(I) complex (8)

Complex **8** was prepared following the same procedures as described for complex **7** except that salt **2** was replaced by **3** (0.41 g, 0.50 mmol) and silver oxide (0.35 g, 1.50 mmol). The silver complex **8** was collected as white precipitate. Yield: 0.42 g (46%). M.p.: 272-277°C. Anal. Calc. for $C_{70}H_{76}Ag_3F_{18}N_{12}P_3$: C, 46.62; H, 4.22; N, 9.32. Found: C, 46.97; H, 4.61; N, 9.56. FTIR

(KBr) υ (cm^{-1}): 3117, 3063 (C_{arom}-H), 2960, 2880 (C_{aliph}-H), 1614, 1477, 1446 (C_{arom}=C_{arom}), 1237 (C_{aliph}-N). ^{13}C NMR (125 MHz, acetonitrile-d_3) δ ppm: 22.9, 24.2, 31.4, 43.9, 49.4, 50.7(CH_2), 60.5(CH); 108.8, 110.6, 121.3, 123.9, 125.0, 133.0(Ar-C), 184.8- 185.2 [(d, J(C^{109}-Ag) = 209.0 Hz and d, J(C-^{107}Ag) = 180.6 Hz], 186.6(C-Ag, br d).

2.2.7. 2-Methylenebenzonitrile substituted tri NHC-Ag(I) complex (9)

Complex **9** was prepared following the same procedures as described for complex **7** except that salt **2** was replaced by **4** (0.21 g, 0.24 mmol) and silver oxide (0.17 g, 0.71 mmol). The silver complex **9** was collected as white precipitate. Yield: 0.24 g (51%). M.p.: 167-171°C. Anal. Calc. for $C_{82}H_{64}Ag_3F_{18}N_{16}P_3$: C, 48.46; H, 3.15; N, 11.03. Found: C, 48.04; H, 2.94; N, 10.71. FTIR (KBr) υ (cm^{-1}): 3121, 3064 (C_{arom}-H), 2941 (C_{aliph}-H), 2224 (C≡N), 1607, 1477, 1446 (C_{arom}=C_{arom}), 1271 (C_{aliph}-N). ^{13}C NMR (125 MHz, acetonitrile-d_3) δ ppm: 44.6, 47.2, 49.2 (CH_2); 111.7, 112.9, 113.9, 117.6, 127.5, 128.7, 129.9, 130.8, 131.2, 133.7, 134.5, 139.8 (Ar-C).

2.2.8. n-Decyl substituted tri NHC-Ag(I) complex (10)

Complex **10** was prepared following the same procedures as described for complex **7** except that salt **2** was replaced by **5** (0.29 g, 0.32 mmol) and silver oxide (0.22 g, 0.96 mmol). The silver complex **10** was collected as white precipitate. Yield: 0.81 g (50%). M.p.: 142-146°C. Anal. Calc. for $C_{90}H_{124}Ag_3F_{18}P_3N_{12}$: C, 50.69; H, 5.82; N, 7.88. Found: C, 50.50; H, 5.37; N, 7.54. FTIR (KBr) υ (cm^{-1}): 3159, 3064 (C_{arom}-H), 2930, 2850 (C_{aliph}-H), 1614, 1496, 1450 (C_{arom}=C_{arom}), 1279 (C_{aliph}-N). ^{13}C NMR (125 MHz, acetonitrile-d_3) δ ppm: 13.1 (CH_3); 22.1, 25.5, 26.0, 27.5, 28.4, 31.3, 38.9, 40.3, 43.2, 45.7, 46.9 (CH_2); 106.6, 108.7, 112.4, 120.9, 121.7, 126.8 (Ar-C).

2.3. In vitro *cytotoxicity studies*

2.3.1. Materials and equipment

The equipment used in this study were, Rosewell Park Memorial Institute (RPMI 1640) cell culture media was obtained from ScienCell, USA. Microplate reader (Epoch, BioTek, USA), Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent, heat inactivated foetal bovine serum (HIFBS) were purchased from GIBCO, UK. Pencillin/streptomycin solution and 5-fluorouracil were purchased from Sigma-Aldrich. Crystal violet, PBS and Tamoxifen were purchased from Sigma-Aldrich. DMEM medium was purchased from Gibco/Life technology, UK. ATCC cell lines were purchased from Bio-Focus Saintifik Sdn Bhd. Malaysia.

Dissecting microscope (Motic, Taiwan), Eppendorf tips (AxyGEN, USA), Eppendorf tube 1.5 mL (Eppendorf, Germany), incubator (Binder Fisher Scientific, Germany), inverted fluorescent microscope (Olympus, Japan), inverted light microscope (Matrix Optic (M) Sdn. Bhd, Japan), Image J software http://rsb.info.nih.gov/ij/, laminar flow (Class II) (ESCO- BSC, Singapore), micro pipette (Eppendorf, USA), refrigerator (SAMSUNG, Japan), serological pipette 10, 5 mL (TPP®,USA), water bath (PROTECH-Electronic, Malaysia), 6-well cell culture plate (Costar Corning, USA).

2.3.2. Cell lines and environmental conditions

Human colorectal tumor (HCT 116) cell lines, human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLA cell line) and human endothelial cell line (EA.hy926 cell line) were purchased from Rockville, MD, USA by Bio-Focus Saintifik Sdn Bhd. Malaysia. HCT 116 cell line was kept in RPMI 1640 cell culture media with the supply of 10% HIFBS and 10% Pencillin/streptomycin solution. Temperature was maintained at 37°C. The cell passage number for HCT-116 was 9-11, MCF-7 was 20-23 and Ea.hy 926 was 5. The cell densities used for seeding cells of HCT-116, MCF-7 and Ea.hy926 were 10,000, 5000 and 20,000 respectively.

2.3.3. Preparation of cell culture

The cell culture was prepared as reported previously [26]. Briefly, the first step in the preparation is the growth of HCT 116 cell lines under incubation conditions. Only those cells which reached about 70-80% level of confluency were selected for plating purposes. The medium used in the plates previously was aspirated and the cells used were washed with phosphate buffer saline solution 2-3 times. After washing with phosphate buffer saline (PBS) solution, trypsin was added evenly on the cell surfaces. The cells were incubated for 1 minute at 37°C. Cell segregation was facilitated by simply tapping the flask containing the cells and then observing them under a 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 was observed (cells were counted using hemocytometer, 10 uL of cell suspension was mixed with 10 uL of tryptophan blue and cells in 4 chambers were counted using microscope) 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.

2.3.4. MTT assay

MTT assay was carried out as reported previously by our group [26]. Briefly, 100 μL of the cells were seeded in all 96 wells of the microplate and incubated overnight for cell attachment under CO_2 incubator. Later on, 100 μL of each test substance was added in each well and the plate was labelled accordingly. Different dilutions of the test substance were prepared to observe dose dependent response. After the addition of the test substance into the plate containing cancer cells, the plate was incubated at 37°C with 5% CO_2 environment for 72 hrs (MTT cell viability assay can be performed for 48-72 hrs, time of experiment depends upon the population doubling time of cells, normally time is allowed for two populations). After 72 hrs of treatment, a volume of 20 μL of MTT reagent was added into each well and it was again incubated for 4 hrs. After this period of incubation, 20 μL of DMSO (MTT lysis solution) was added in each well. The Plates were further incubated for 5 minutes under the same environment of incubation. Finally, absorbance was taken at 570 nm and 620 nm using a microplate reader (Epoch, BioTek, USA). The data was analyzed for the cell viability and % inhibition of proliferation of test substances. The results were presented as percent viability compared to the negative control (mean ± SD, n=3) [28].

2.3.5. Statistical analysis

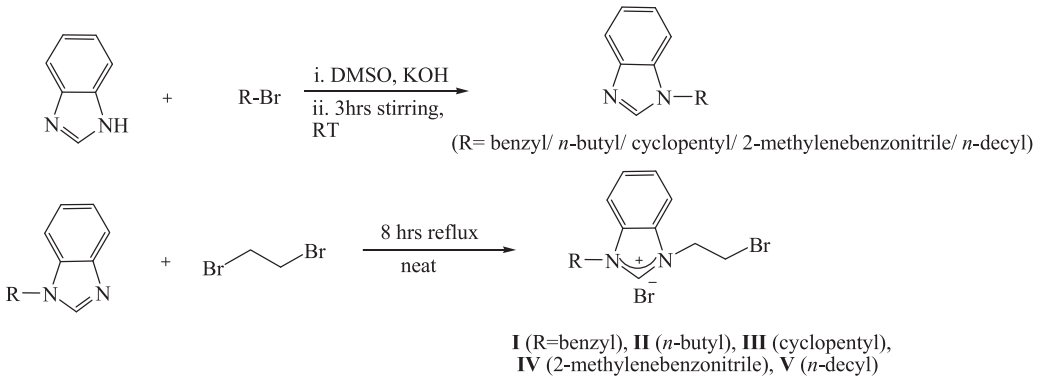
The percentage inhibition of proliferation data was analyzed for significance by two-way ANOVA and then compared by Bonferroni tests using GraphPad Prism Software, Version 5.01. One-way ANOVA was performed to study the overall mean percentage of inhibition of proliferation, cell migration and colony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

3. Results and discussion

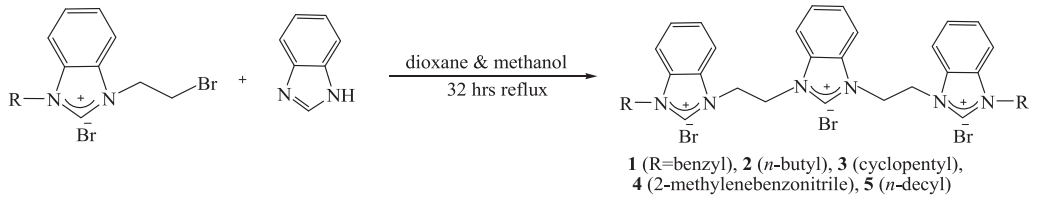
3.1. Synthesis and characterization

The tri-NHC proligands (**1**-**5**) and respective trinuclear silver(I) complexes (**6**-**10**) were synthesized by a multistep designed scheme first reported by our group (Scheme 1-3) Figure Scheme. 2[10]. The structure of the proligands (**2**-**5**) is proposed to be similar to the reported single crystal structure of salt **1** [10], supported by ^{1}H-, ^{13}C-NMR, IR and elemental analysis.

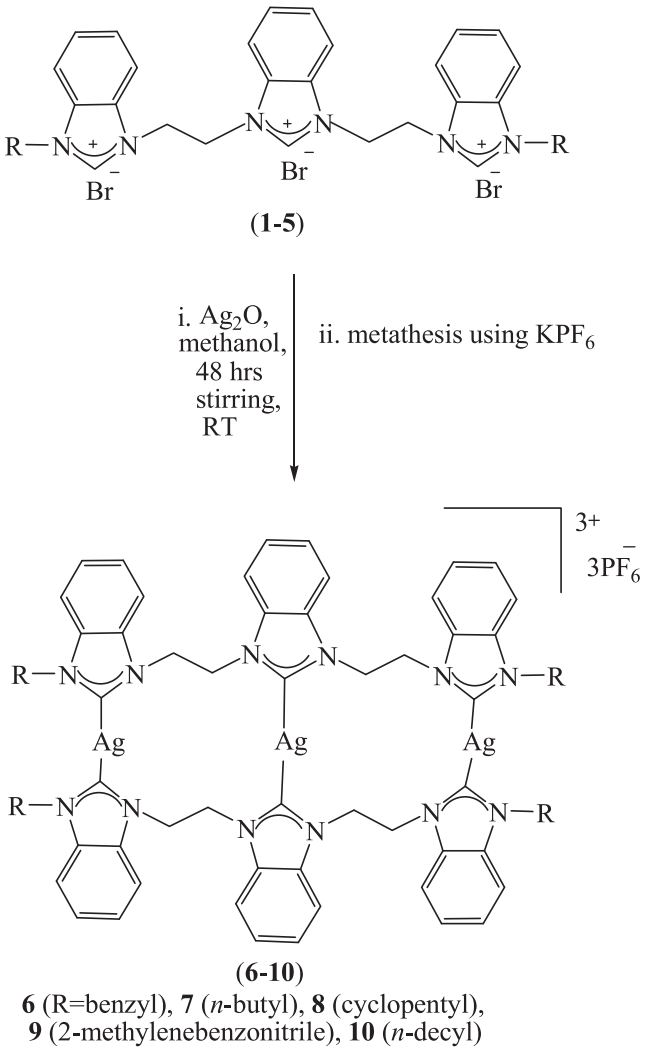
The ^{1}H- and ^{13}C-NMR provide characteristic evidence for the successful synthesis of the desired tris benzimidazolium salts and the respective Ag(I) complexes. In ^{1}H NMR of tris benzimidazolium



Scheme 1. Synthesis of 3-(2-bromoethyl)-1-substitutedbenzimidazolium bromides (**I-V**).



Scheme 2. Synthesis of tris benzimidazolium bromides (**1-5**).



Scheme 3. Synthesis of tri-NHC trinuclear silver(I) complexes (**6-10**).

salts (**1-5**), the acidic protons of the three pre-carbenic centres exhibit two singlets in the most downfield region within 10.1-10.3 ppm [18], as these three protons are under two different chemical environments. Resonances of all the bridging dimethylene protons appear in the form of a singlet around 5.2 ppm in all of the salts which signify the non-rigidity of the structures [29]. In addition to the characteristic and common proton resonances of the core structures, the proton resonances of the terminal substituents in each salt appear in their expected regions, thus signifying the formation of the desired salts. In ^{13}C NMR the most distinctive resonances are displayed by the pre-carbenic carbons (NCHN) in the most deshielded region in the range of 142-145 ppm [30,31].

The generation of carbene centres is also supported by ^{1}H NMR spectra of each complex as no resonances could be observed for the acidic protons around 10 ppm in all spectra [32,33]. The resonances of the rest of the protons of the ligands are broadened and overlapped, which implies that all the tri-NHC trinuclear Ag(I) complexes have a highly flexible structure as previously reported [10]. The evidence for the formation of $C_{carbene}$-Ag(I) bond is provided by ^{13}C-NMR analysis in which the complexes show doublets of doublets between 183-199 ppm with the coupling constants of 180.6 Hz for C-^{107}Ag and 209 Hz for C-^{109}Ag for the $C_{carbene}$-Ag(I) coupling [34,35]. The Ag-carbene coupling was only observed in the ^{13}C-NMR spectrum of compounds **7** and **8** to any resolvable degree while the compounds **9** and **10** had carbene resonances that were sufficiently broadened to be undetectable. The structures of all the tri-NHC trinuclear silver(I) complexes could not be further supported by X-ray crystallography since all efforts to grow single crystals were unsuccessful. As reported before [11], we postulate that the tri-NHC trinuclear silver(I) complexes in this work exist as trinuclear structures from two tri-NHC ligands with linear geometry, the evidence of structural symmetry in the NMR spectrum provides clear support of the proposed structure. The coordination of three carbene centres with three silver(I) ions in a tri-metal diligand arrangement is provided by elemental analysis which supports the stoichiometry $[Ag_3L_2]\cdot 3PF_6$ for the complexes *(vide supra)* (**6-10**).

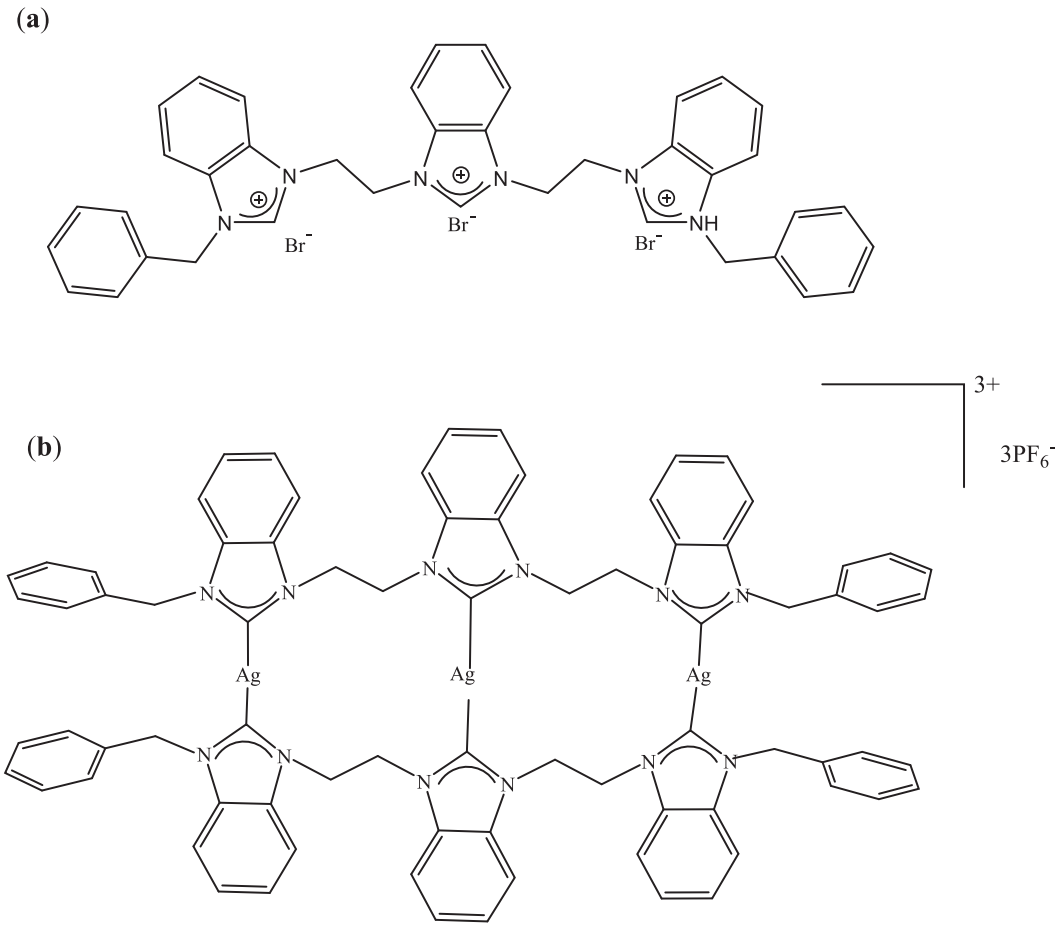


Fig. 1. The previously reported tris azolium (benzimidazole/imidazole) salts (a); and the respective tri-NHC trinuclear silver(I)- complexes (b).

3.2. In vitro *cytotoxic activities of tri-NHC trinuclear silver(I) complexes and their respective proligands*

3.2.1. In vitro *cytotoxic activities of tri-NHC trinuclear silver(I) complexes and their respective proligands against the human colon cancer (HCT-116) cell line*

It has been reported in the previous studies that the mono-NHC trinuclear Ag(I) imidazolium complexes showed weak anticancer activity against the human colon cancer cell lines [36]. Based on the observations and results of the anticancer activity of mono- and di- NHC Ag(I) complexes and their respective proligands [24,25], the anticancer activity of the trinuclear Ag(I)-NHC complexes derived from tris benzimidazolium salts is expected to be enhanced by increasing the number of silver centres and the NHC units in the complexes. In order to explore the cytotoxic activity of the proligands and complexes containing three NHCs units and three Ag(I) ions, aforementioned proligands (**1**-**5**) and their respective complexes (**6**-**10**) were synthesized successfully in a programmed and schematic way. The tri-NHC trinuclear Ag(I) complexes have not been studied previously for their antitumor activity and has yet to be assessed, thus implying that the antitumor behaviour of these compounds could not be predicted. Thus, in order to evaluate the anticancer potential of the tri-NHC trinuclear Ag(I) complexes, the proligands (**1**-**5**) and the complexes (**6**-**10**) containing different substituents were tested against the human colon cancer cell line, HCT116. In the initial screening, all the synthesized proligands and the complexes were evaluated for the IC_{50} values. Herein, most of the compounds are inactive on HCT116 except for the proligand **5** and complexes **9** and **10** (Table 1). This effect of the compounds is in accordance with the reported results of mono-NHC trinuclear Ag(I) complexes [36]. The non-active tri-NHC trinuclear Ag(I) complexes and the proligands may be attributed to the different substitutions within these compounds. The results in the present study are in accordance with the earlier findings that the substitution patterns/steric bulk on the benzimidazolium ring has a major influence on the anticancer potential [37] although a few other studies have reported that the flexibility, nature of the NHC ligands, hydrogen bonding capabilities of the NHC-Ag(I), the number of silver centres and the charge can influence the anticancer potential [38]. It can be deduced from the IC_{50} values that the inactivity of these proligands (**1**-**4**) and their respective complexes (**6**-**8**) might be caused by the steric bulk and an enhanced stability of the molecules that might inhibit the activity by preventing an adequate release of silver ions. The proligand **5** and its respective complex **10** containing long, 10-carbon chains, however, showed IC_{50} values of 14.98 μM and 0.26 μM, respectively, as compared to that of the standard drug Table 1.

The results support the fact that incorporation of long carbon side chains may facilitate drug absorption and increase the cytotoxic activity of the tri NHC compounds, a similar phenomenon observed in the mono- and di-NHC proligands and their respective complexes [24,25]. Complex **9** containing 2-methylenebenzonitrile has an IC_{50} approximately three times smaller than that of 5-FU, thus implying that a functional group does play a role in cytotoxic activity. The activity of this complex is in accordance with those of the previously reported nitrile functionalized anticancer drugs in *in vitro* and *in vivo* experiments on different cell lines [39,40].

Table. 1
IC_{50} values of the proligands (**1**-**5**) and their respective complexes (**6**-**10**) on HCT 116 and EA.hy926 using the standard drug 5-FU (IC_{50} = 10.2 μM), MCF-7 using the standard drug Tamoxifen (IC_{50} = 7.5μM) and HeLa cell lines using the standard drug 5-FU (IC_{50} = 10.2 μM) as a positive control.

Proligands	Tri NHC-Ag(I) complexes	IC_{50} (μM) HCT 116 5-FU (IC_{50} = 10.2 μM)		EA.hy926		IC_{50} (μM) MCF-7 Tamoxifen (IC_{50} = 7.5μM)		IC_{50} (μM) HeLa cell lines 5-FU (IC_{50} = 10.2 μM)	
1	**6**	NA	NA	NA	6.16	NA	7.78	NA	2.77
2	**7**	NA	NA	NA	12.3	NA	0.02	NA	8.65
3	**8**	NA	NA	NA	9.4	NA	14.83	NA	0.40
4	**9**	NA	3.2	NA	9.38	NA	18.66	NA	10.89
5	**10**	14.98	0.26	35	11	1.42	0.27	5.43	1.51

$^{\pm}$*NA = not active*

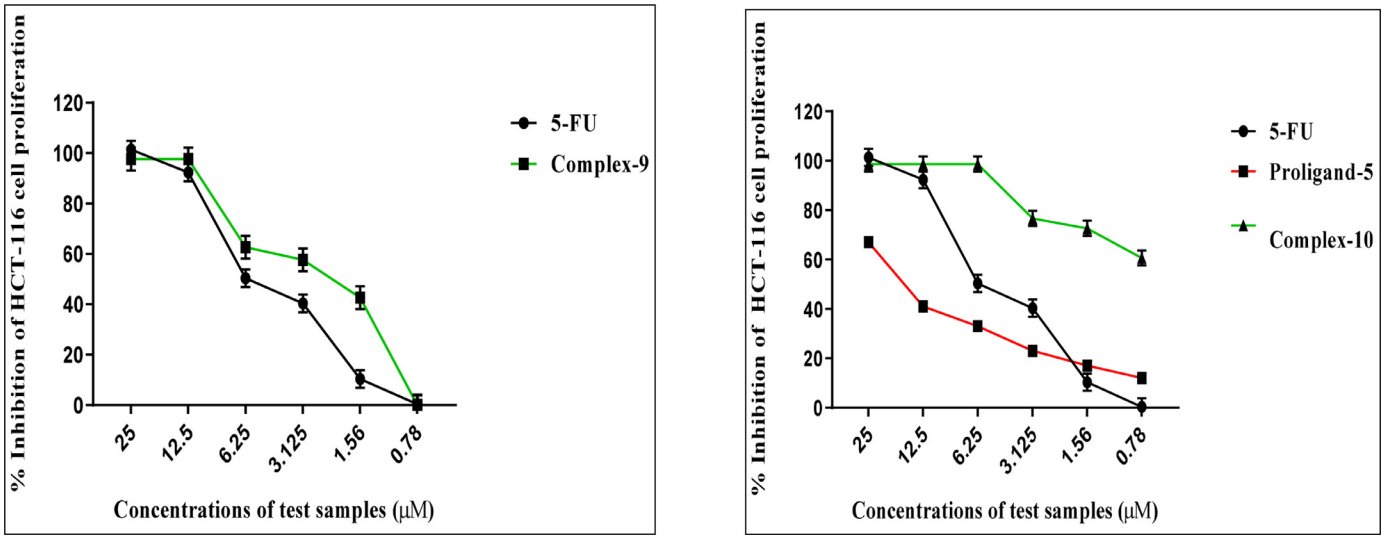


Fig. 2. Dose dependent proliferative effects of complex **9, 10** and proligand **5** against the human colon cancer cell line in comparison with the standard drug 5-FU.

The IC_{50} values of the synthesized tri-NHC compounds (proligands **1**-**4** and complexes **6**-**8**) indicate that these compounds are inactive against the human colon cancer cell lines, hence their effects on the cell proliferation are not considered in this study. The complex **9** containing a nitrile functional group shows a dose dependent inhibition in the proliferation of cancer cells on HCT116 (Fig. 2). Even though proligand **5** shows a percentage inhibition of proliferation on HCT116 cell lines, this effect however is less prominent than that of the standard drug 5-FU. However, an exploration of these responses using complex **10** demonstrates a significant higher percentage inhibition in cell proliferation in low, medium and high doses as compared to the respective proligand and the standard drug 5-FU (Fig. 2). The photomicrographic images of HCT116 cell lines and EA.hy926 cell lines after treated with proligands **5** and complexes **9** and **10** are shown in Fig. 3. These compounds are found to be less toxic on the normal cell lines (EA.hy926), but relatively toxic on the HCT 116 cell line.

3.2.2. In vitro *cytotoxic activities of tri-NHC trinuclear silver(I) complexes and their respective proligands against human breast cancer cell line (MCF-7)*

The results of cytotoxic studies of the tri-NHC trinuclear silver(I) complexes and the respective proligands on HCT116 indicate that these compounds are inactive, hence in order to find whether these compounds are also inactive on other cancer cell lines, they were further evaluated against breast cancer cell line (MCF-7). As reported in the literature compounds which are inactive on one cancer cell line are found to be active on the other cancer cell lines [41]. Unlike the IC_{50} values on HCT116 cell lines, in which only the proligand **5** and the complexes **9** and **10** are active, the IC_{50} val-

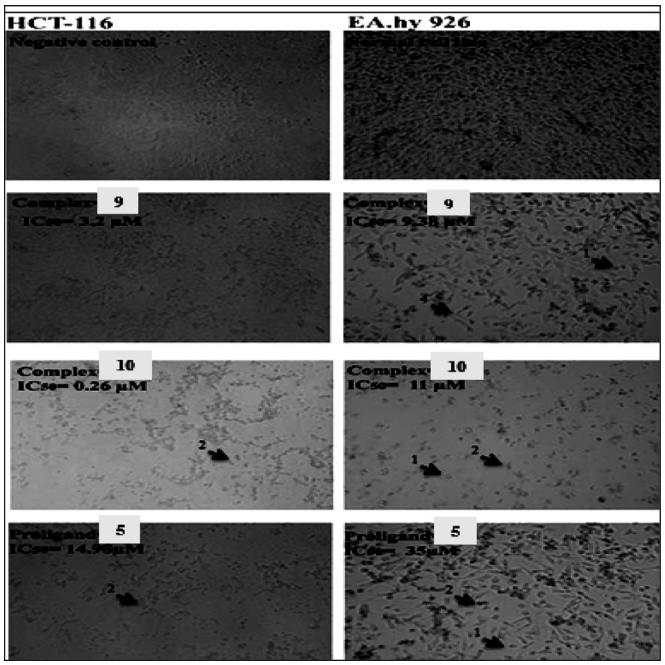


Fig. 3. Images of human colorectal cancer cell lines and human endothelial cell lines showing the effects of proligands **5** and complexes **9** and **10**. (cell images were taken under a light microscope at × 200 magnification with a digital camera at 48 hours after treatment). Arrows points out the altered morphology of cells (1 = cell shrinkage, 2 = dead cells, 3 = detached floating cells not seen, 4 = loss of contact between cells).

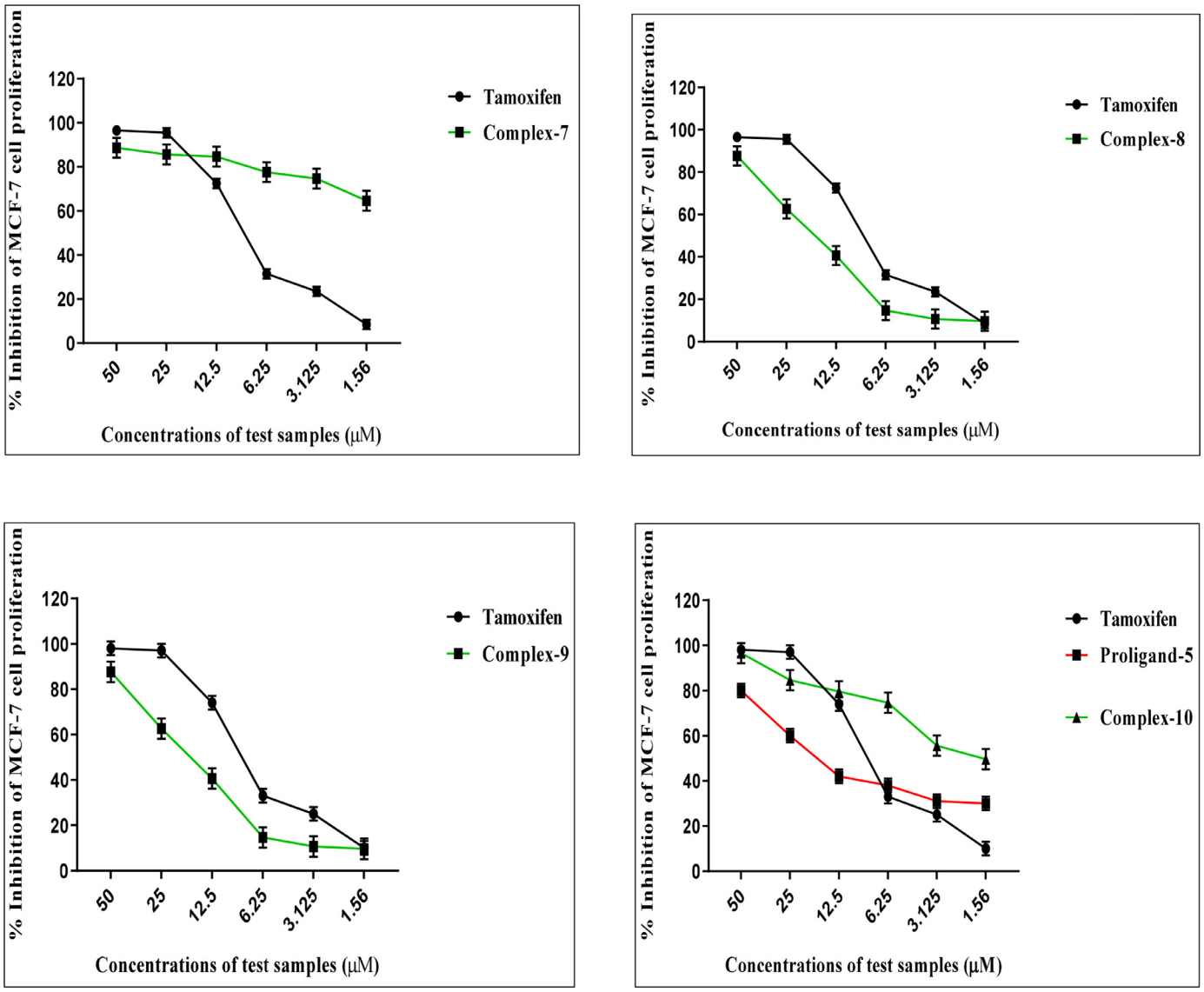


Fig. 4. Dose dependent antiproliferative effects of complexes **7, 8, 9, 10** and proligand **5** against breast cancer cell line in comparison with the standard drug, Tamoxifen.

ues of all the tri-NHC compounds show large variability, though only proligand **5** and all trinuclear complexes show activity against MCF-7 (Table 1).

It is of great interest that proligand **5** and the respective complex **10** show an IC_{50} of 5.2 and 28 times, respectively, lesser when compared to that of the standard drug, Tamoxifen (IC_{50} = 7.5μM). Furthermore, the antiproliferation activity of the proligand **5** and complexes (**6**-**10**) with different doses in the range of 1.56-50 μM, along with the same dilution of the standard drug Tamoxifen were also examined. For complex **6**, although the IC_{50} value is comparable to that of the standard drug, the antiproliferation activity is not promising. Besides, complex **7** with *n*-butyl side chains shows a dose dependent response (Fig. 4). This complex displays antiproliferative activity on the breast cancer cell lines at lower, medium and higher doses and these responses are higher in percentage as compared to that of Tamoxifen (Fig. 4). Similarly, complex **8** shows significant percentage inhibition of proliferation but lower than that of the standard drug. This observation could be due to the presence of steric bulk of the substituent group in complex **8**. Complex **9**, containing 2-methylene benzonitrile showed dose dependent responses of antiproliferation activity in a fashion similar to that of complex **8**.

Another significant result is that the complex **10** shows greater percentage inhibition of proliferation as compared to that of the respective proligand **5**, although both show dose dependent responses on MCF-7. All complexes show cytotoxicity against the breast cancer cells which is in accordance with the results of a previous study which reports the imidazolium-based mononuclear silver(I)-NHC complexes as anticancer agents against breast cancer cell lines [41].

3.2.3. In vitro *cytotoxic activities of tri-NHC trinuclear silver(I) complexes and their respective proligands against the human epitheloid cervix carcinoma (HeLa) cell line*

All the synthesized proligands and their respective tri-NHC trinuclear Ag(I) complexes were also investigated for their activity on HeLa cell lines, in which their IC_{50} values were also calculated. All complexes show an IC_{50} values similar to those observed on the MCF-7 cell line. The IC_{50} values on HeLa cell line vary depending upon the type of substituents present in the structure moiety (Table 1).

Interestingly, among all the studied proligands, only proligand **5** shows activity on the HeLa cell line with an IC_{50} of 5.43 μM, which is an indicative of a high lipophilicity of this proligand.

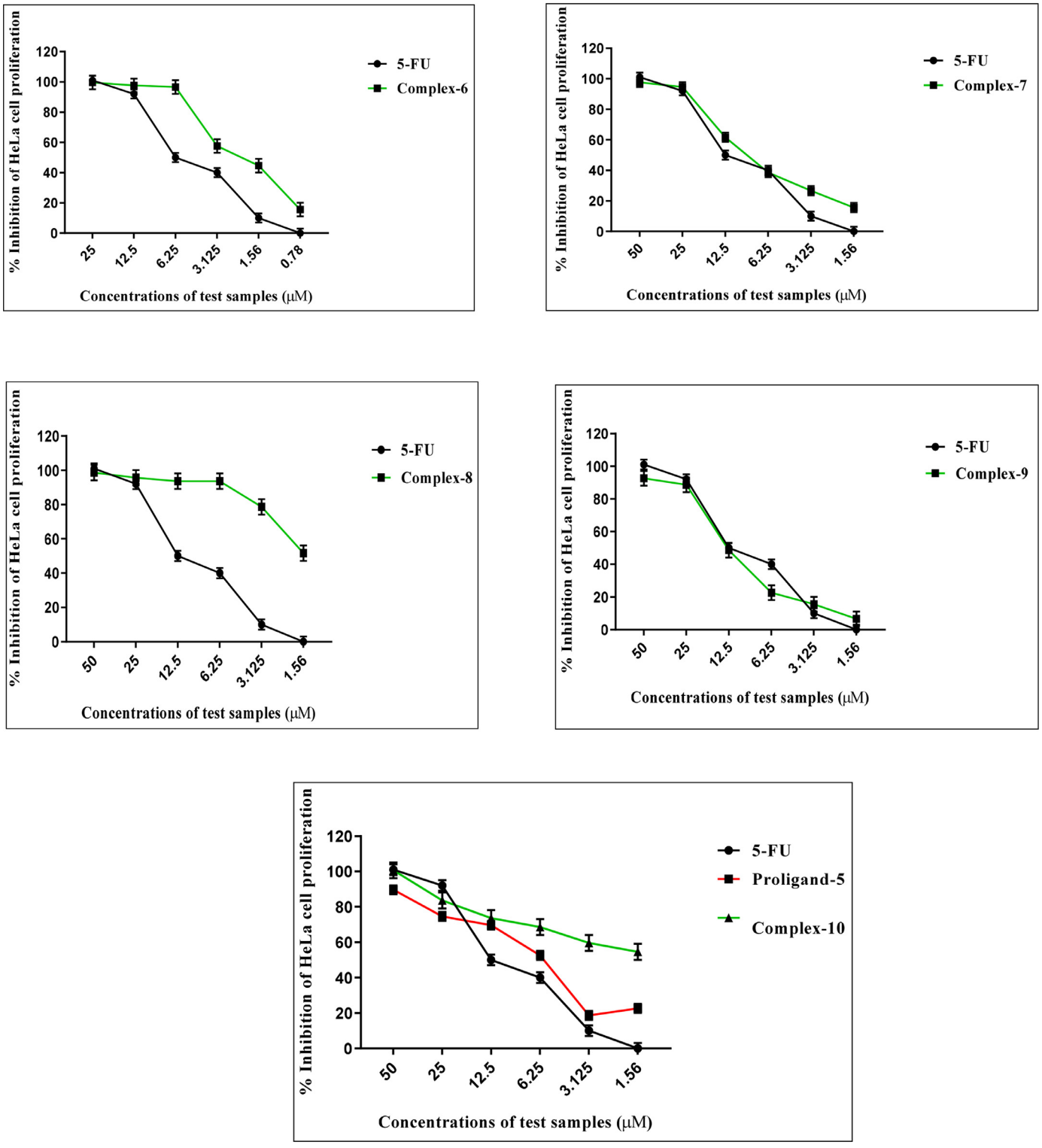


Fig. 5. Dose dependent antiproliferative effects of complex **6, 7, 8, 9, 10** and proligand **5** in comparison with the standard drug 5-FU.

Aforementioned, increasing the carbon chain length will enhance the lipophilicity, thereby facilitating a greater bioavailaibility of the proligand in the cell [42]. The active compounds, after screening for their IC_{50} values, were evaluated for their proliferation inhibition activities against the human epitheloid cervix cancer cell line, keeping in view the fact that the silver complexes are reported before to have antiproliferative potential against the HeLa cell line [43]. Complex **6**, shows dose dependent responses that are better than those of the standard drug at lower and medium doses, but comparable to that of the latter at the highest dose (Fig. 5). Complex **7**, exhibits a dose dependent inhibition of proliferation of the HeLa cancer cells. Noteworthy, the respective proligand is inactive to HeLa, thus suggesting that not only the NHC moiety is responsible for the overall responses but such anticancer activities can be attributed to the additional silver ions present in the complex. Complex **8** shows greater responses in terms of percentage inhibi-

tion of proliferation at lower and medium doses but comparable to 5-FU at higher dose while complex **9** shows inhibition of proliferation comparable to the similar standard drug (Fig. 5). The only proligand in this series that shows activity on the HeLa cell lines is the proligand **5** that shows higher percentage inhibition of proliferation at lower and medium doses as compared to the standard drug used. Additionally, complex **10** shows higher percentage inhibition of proliferation at all doses compared to the respective proligand **5**. At the maximum doses (25 and 50 μM), the percentage inhibition of proliferation of complex **10** and 5-FU are more comparable than proligand **5**. All the complexes **6**-**10** show potential cytotoxicity against the cervical cancer which is in contrast with the previous findings of mono-NHC mononuclear Ag(I) complexes on the same cell lines [41]. This thus suggesting that anticancer activity can be attributed to both the additional silver ions present in the complex and the NHC moiety. The observed phenomenon of increased cytotoxicity of the NHC-Ag(I) complexes, in comparison to their respective proligands is in line with the previously reported data [44]. The mechanism of cytotoxicity of these tri NHC-Ag(I) complexes can be explored in future studies.

The cytotoxicity results of the present study on tri NHC-Ag(I) complexes when compared to similar substituted mono, di and tetra-NHC-Ag(I) complexes previously studied by our group indicates a decrease in activity for tri and tetra NHC-Ag(I) complexes. The factors responsible for such an unexpected decrease in anticancer potential are the poor solubility of these bulkier complexes that reduces their bioavailability and efficacy, in addition an inadequate release of Ag(I) ions due to an increase in the NHC units in the ligand molecule that might tightly hold the silver ions.

4. Conclusion

This study accounts for the successful synthesis and characterization of tris benzimidazolium bromide salts (**1**-**5**) and their respective trinuclear silver(I) complexes (**6**-**10**). The cytotoxic potential of the tested compounds (**1**-**10**) determined by IC_{50} values and antiproliferative activity against human colon, human breast and human epitheloid cervix cancer cell lines revealed these compounds to be more cytotoxic towards breast cancer and cervical cancer cell lines and selectively toxic against human colon cancer cell line. On all studied cell lines, the complexes are found to be cytotoxic compared to their proligands, which signifies the ability of silver ions to kill cancer cells. Among all the active compounds, complex **10** containing *n*-decyl substituent exhibits pronounced activity on the selected cell lines which imply the role of enhanced lipophilicity on drug absorption in addition to the silver ions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank USM for financial support through funding 1001/PKIMIA/8011089.

References

[1] J. Cheng, L. Wang, P. Wang, L. Deng, Chem. Rev. 118 (2018) 9930–9987.
[2] T. Weskamp, V.P.W. Bohm, W.A. Herrmann, J. Organomet. Chem. 600 (2000) 12–22.
[3] F.E. Hahn, Chem.Rev. 118 (2018) 9455–9456.
[4] X. Hu, I. Castro-Rodriguez, K. Olsen, K. Meyer, Organomet. 23 (2004) 755–764.
[5] A. Rit, T. Pape, F.E. Hahn, J. Am. Chem. Soc. 132 (2010) 4572–4573.
[6] X. Hu, I. Castro-Rodriguez, K. Meyer, J. Am. Chem. Soc. 125 (2003) 12237–12245.
[7] E. Mas-Marza, M. Poyatos, M. Sanau, E. Peris, Inorg. Chem. 43 (2004) 2213–2219.
[8] C.E. Ellul, G. Reed, M.F. Mahon, S.I. Pascu, M.K. Whittlesey, Organometallics 29 (2010) 4097–4104.
[9] Y.W. Zhang, R. Das, Y. Li, Y.Y. Wang, Y.F. Han, Chem.-A Eur. J. 25 (2019) 5472–5479.
[10] T. Fatima, R.A. Haque, M.R. Razali, J. Mol. Struct. 1141 (2017) 346–350.
[11] S. Gonell, M. Poyatos, E. Peris, Angewandte Chemie 52 (2013) 7009–7013.
[12] A. Biffis, G.G. Lobbia, G. Papini, M. Pellei, C. Santini, E. Scattolin, C. Tubaro, J. Organomet. Chem. 693 (2008) 3760–3766.
[13] X. Hu, Y. Tang, P. Gantzel, K. Meyer, Organometallics 22 (2003) 612–614.
[14] S.Y. Hussaini, R.A. Haque, M.R. Razali, J. Organomet. Chem. 882 (2019) 96–111.
[15] S. Budagumpi, R.A. Haque, S. Endud, G.U. Rehman, A.W. Salman, Eur. J. Inorg. Chem. 2013 (2013) 4367–4388.
[16] S. Kankala, N. Thota, F. Bjorkling, M.K. Taylor, R. Vadde, R. Balusu, Drug Develop. Res. 80 (2019) 188–199.
[17] S.A. Patil, S.A. Patil, R. Patil, R.S. Keri, S. Budagumpi, G.R. Balakrishna, M. Tacke, Future Med. Chem. 7 (2015) 1305–1333.
[18] P.O. Asekunowo, R.A. Haque, M.R. Razali, Rev. Inorg. Chem. 37 (2017) 29–50.
[19] S. Tan, Y. Yan, P. Lee, K. Lim, Future Med. Chem. 2 (2010) 1591–1608.
[20] L. Eloy, A.S. Jarrousse, M.L. Teyssot, A. Gautier, L. Morel, C. Jolivalt, T. Cresteil, S. Roland, Chem. Med. Chem. 7 (2012) 805–814.
[21] J. Dinda, T. Samanta, A. Nandy, K.D. Saha, S.K. Seth, S.K. Chattopadhyay, C.W. Bielawski, New J. Chem. 38 (2014) 1218–1224.
[22] C. Saturnino, I. Barone, D. Iacopetta, A. Mariconda, M.S. Sinicropi, C. Rosano, A. Campana, S. Catalano, P. Longo, S. Andò, Future Med. Chem. 8 (2016) 2213–2229.
[23] T. Fatima, R.A. Haque, M.A. Iqbal, A. Ahmad, L.E.A. Hassan, M. Taleb-Agha, M.B.K. Ahamed, A.A. Majid, M.R. Razali, J. Organomet. Chem. 853 (2017) 122–135.
[24] T. Fatima, R.A. Haque, M.R. Razali, A. Ahmad, M. Asif, M.B. Khadeer Ahamed, A.M.S. Abdul Majid, Appl. Organomet. Chem 31 (2017) e3735.
[25] T. Fatima, R.A. Haque, M.R. Razali, A. Ahmad, M.A. Iqbal, M. Asif, M.B.K. Ahamed, A. Abdul Majid, J. Coord. Chem. 69 (2016) 3367–3383.
[26] T. Fatima, R.A. Haque, M.A. Iqbal, A. Ahmad, L.E.A. Hassan, M. Taleb-Agha, M.B.K. Ahamed, A.M.S.A. Majid, M.R. Razali, J. Organomet. Chem. 853 (2017) 122–135.
[27] T. Fatima, R.A. Haque, M.A. Iqbal, M.R. Razali, J. Organomet. Chem. 831 (2017) 50–54.
[28] A.F. Aisha, K.M. Abu-Salah, Z. Ismail, A.M.S.A. Majid, BMC Complement. Altern. Med. 12 (2012) 104.
[29] O. Sanchez, S. González, M. Fernández, A.R. Higuera-Padilla, Y. Leon, D. Coll, A. Vidal, P. Taylor, I. Urdanibia, M.C. Goite, Inorg. Chim. Acta 437 (2015) 143–151.
[30] B. Yigit, Y. Gok, I. Ozdemirr, S. Gunal, J. Coord. Chem. 65 (2012) 371–379.
[31] C. O'Beirne, H.T. Althani, O. Dada, J. Cassidy, K. Kavanagh, H. Muller-Bunz, Y. Ortin, X. Zhu, M. Tacke, Polyhedron 149 (2018) 95–103.
[32] S. Patil, K. Dietrich, A. Deally, B. Gleeson, H. Muller Bunz, F. Paradisi, M. Tacke, Helvetica Chim. Acta 93 (2010) 2347–2364.
[33] C. O'Beirne, N.F. Alhamad, Q. Ma, H. Muller-Bunz, K. Kavanagh, G. Butler, X. Zhu, M. Tacke, Inorg. Chim. Acta 486 (2019) 294–303.
[34] B. Bildstein, M. Malaun, H. Kopacka, K. Wurst, M. Mitterback, K.-H. Ongania, G. Opromolla, P. Zanello, Organometallics 18 (1999) 4325–4336.
[35] J.C. Garrison, W.J. Youngs, Chem. Rev. 105 (2005) 3978–4008.
[36] A.W. Salman, R.A. Haque, Eur. J. Chem. 7 (2016) 115–120.
[37] R.A. Haque, S.Y. Choo, S. Budagumpi, M.A. Iqbal, A.A.-A. Abdullah, Eur. J. Med. Chem. 90 (2015) 82–92.
[38] 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.
[39] F. Iduna, C. Jindrich, M. Martin, C.S. Lara, H. Ralf, N.K. Breandan, L.R. Alison, H. Frauke, L. Grainne, J.Q. Susan, M. Ian, T. Matthias, Lett. Drug Design Discov. 9 (2012) 815–822.
[40] H.Z. Zulikha, R.A. Haque, S. Budagumpi, A.M.A. Majid, Inorg. Chim. Acta 411 (2014) 40–47.
[41] M.Doug A, H.Khadijah M, P.Matthew J, D.Andrew J, Y.Yang H, Y.Wiley J, Metal-Based Drugs, 2008 (2008).
[42] S.N. Riduan, Y. Zhang, Chem. Soc. Rev. 42 (2013) 9055–9070.
[43] R. Sukirtha, K.M. Priyanka, J.J. Antony, S. Kamalakkannan, R. Thangam, P. Gunasekaran, M. Krishnan, S. Achiraman, Process Biochem. 47 (2012) 273–279.
[44] N.A. Johnson, M.R. Southerland, W.J. Youngs, Molecules 22 (2017) 1263.