SYNTHESIS, CHARACTERIZATION, AND *IN VITRO* ANTICANCER ACTIVITIES OF BENZYL-FUNCTIONALIZED BENZIMIDAZOLIUM SALTS AND THEIR SILVER(I)-*N*-HETEROCYCLIC CARBENE COMPLEXES

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by

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LIST OF SYMBOLS AND UNITS

α	Alpha
δ	Chemical shift
J	Coupling constant
°C	Degree Celsius
g	gram
kV	Kilovolt
L	Liter
L/h	Litres per hour
m/z	Mass-to-charge ratio
MHz	Megahertz
µg	Microgram
µg/mL	Microgram per millilitre
µm	Micrometre
µM	Micromolar
mg	Milligram
mL	Millilitre
mm	Millimeter
mmol	Millimole
nm	Nanometer
ppm	Parts per million
cm^{-1}	Per centimetre

%	Percentage
π	Pi
σ	Sigma
V	Voltage
v/v	Volume/volume ratio
v cm^{-1}	Wavenumber

LIST OF ABBREVIATIONS

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
AChE	Acetylcholinesterase
ATCC	American Type Culture Collection
AR	Analytical reagent
ATR-FT-IR	Attenuated total reflection-fourier-transform infrared
Br	Bromine
BChE	Butyrylcholinesterase
Cald.	Calculated
C. albicans	*Candida albicans*
C	Carbon
CHN	Carbon nitrogen hydrogen
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
CND	Center for Nano-materials and Displays
CDR	Centre for Drug Research
Cl	Chlorine
$CDCl_3$	Chloroform
CN	Cyano
Acetone-d_6	Deuterated chloroform
DMSO-d_6	Deuterated dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
DEPT	Distortionless enhancement by polarization transfer

d	Doublet
dd	Doublet of doublets
DMEM	Dulbecco's Modified Eagle Medium
ESI-MS	Electrospray ionization mass spectrometry
E. faecalis	*Enterococcus faecalis*
E. coli	*Eschericia coli*
FBS	Fetal bovine serum
F	Fluorine
FT-IR	Fourier-transform infrared spectroscopy
FT-NMR	Fourier-transform nuclear magnetic resonance spectroscopy
IC_{50}	Half maximal inhibitory concentration
MCF-7	Human breast cancer cell lines
MDA-MB-231	Human breast cancer cell lines
Caki-1	Human cancerous renal cell lines
hCAI	Human carbonic anhydrase I
hCAII	Human carbonic anhydrase II
HeLa	Human cervical cancer cell lines
KB-V1/Vbl	Human cervix carcinoma cell lines
A549cisR	Human cisplatin-resistant lung cancer cell lines
A2780/DDP	Human cisplatin-resistant ovary adenocarcinoma cell lines
HCT116	Human colorectal adenocarcinoma cell lines
HCT119	Human colorectal adenocarcinoma cell lines
HT29	Human colorectal adenocarcinoma cell lines

LoVo	Human colorectal adenocarcinoma cell lines
K-562	Human erythromyeloblastoid leukemia cell lines
HL-60	Human leukemia cell lines
MV-4-11	Human leukemia cell lines
A549	Human lung cancer cell lines
H1299	Human lung cancer cell lines
KB3-1	Human nasopharyngeal epidermis carcinoma cell lines
Hs27	Human normal skin fibroblast cell lines
TOV21G	Human ovarian cancer cell lines
SK-OV3	Human ovary adenocarcinoma cell lines
OVCAR-8	Human ovary adenocarcinoma cell lines
A2780	Human ovary adenocarcinoma cell lines
DU-145	Human prostate cancer cell lines
H	Hydrogen
^{1}H-NMR	Hydrogen-1 nuclear magnetic resonance
LC-MS	Liquid chromatography mass spectrometry
L. monocytogenes	*Listeria monocytogenes*
m	*meta*
metal-NHC	Metal-*N*-heterocyclic carbene
MIC	Minimum inhibitory concentration
m	Multiplets
NHC	*N*-heterocyclic carbene
NO	Nitro

N	Nitrogen
NMR	Nuclear magnetic resonance
1D	One-dimensional
o	*ortho*
p	*para*
PTFE	Polytetrafluroethylene
KPF_6	Potassium hexafluorophosphate
KOH	Potassium hydroxide
P. aeruginosa	*Pseudomonas aeruginosa*
quint	Quintet
S. typhimurium	*Salmonella typhimurium*
SI	Selectivity index
$Ag_2C_2H_3O_2$	Silver Acetate
Ag_2CO_3	Silver Carbonate
Ag_2O	Silver(I) oxide
s	Singlet
SD	Standard deviation
S. aureus	*Staphylococcus aureus*
SAR	Structure-activity relationship
TLC	Thin layer chromatography
T	Triplet
2D	Two-dimensional
UPLC-MS	Ultra-performance liquid chromatography mass spectrometry

UV-Vis	Ultraviolet-visible
USM	Universiti Sains Malaysia
α-Gly	α-glycosidase

LIST OF APPENDICES

SINTESIS, PENCIRIAN, PENILAIAN *IN VITRO* GARAM BENZIMIDAZOLIUM TERTUKARGANTI BENZIL DAN KOMPLEKS ARGENTUM(I)-*N*-HETEROSIKLIK KARBENA

ABSTRAK

Kanser kekal sebagai cabaran kesihatan global yang berterusan, memerlukan agen terapeutik yang inovatif. Ubat berasaskan logam, terutamanya yang mengandungi ion argentum(I), menunjukkan potensi yang menjanjikan kerana kereaktifan tinggi dan keupayaan untuk membentuk kompleks stabil dengan molekul biologi, memberikan kelebihan berbanding rawatan tradisional. Ubat kemoterapi semasa sering menyebabkan toksisiti yang teruk dan mengalami rintangan ubat, yang mendorong pembangunan sebatian baru dengan keberkesanan dan kepilihan yang lebih baik. Dalam kajian ini, dua siri baru garam benzimidazolium tidak simetri berfungsi benzil dan kompleks argentum (I)-karbena *N*-heterosiklik (NHC) mereka telah disediakan. Garam benzimidazolium, **C2-C11**, disintesis melalui tindak balas *N*-pengalkilan antara 1-desilbenzimidazol dengan benzil/tertukarganti benzil bromide, diikuti dengan deprotonasi*in situ* dengan argentum oksida (Ag_2O) dan tindak balas metatesis dengan kalium heksafluorofosfat (KPF_6), untuk menghasilkan kompleks argentum(I)-NHC, **C12-C21**. Sebatian-sebatian ini dicirikan menggunakan spektroskopi Fourier-transform inframerah (FT-IR) dan spektroskopi Fourier-transform nuklear magnetik resonan (FT-NMR), kromatografi cair-spektrometri jisim (LC-MS), kromatografi cair ultra-prestasi fasa terbalik (UPLC-MS) dan analisis unsur CHN. Sitotoksisiti *in vitro* daripada sebatian-sebatian ini terhadap sel kanser serviks

manusia (HeLa), sel kanser payudara (MCF-7), dan sel fibroblas kulit normal (Hs27) dinilai melalui ujian 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida (MTT). Semua sebatian menunjukkan perencatan pertumbuhan yang ketara terhadap sel-sel yang diuji, melebihi prestasi ubat antikanser klinikal, etoposid. Fluorinasi meningkatkan kesan sitotoksik terhadap sel HeLa dan Hs27 secara selektif, tetapi tidak mempunyai kesan sitotoksik yang dipertingkatkan terhadap sel MCF-7. Substituen *para* (CH_3, Cl, Br, CN, NO_2) pada bahagian benzil memodulasi potensi dan selektiviti terhadap sel-sel yang diuji, dengan derivatif tersubstitusi kumpulan metil menunjukkan aktiviti lebih tinggi terhadap sel MCF-7 dan ketoksikan lebih rendah terhadap sel Hs27. Penggabungan ion argentum(I) dengan ketara meningkatkan keberkesanan sitotoksik dan selektiviti kompleks argentum(I)-NHC secara signifikan, dengan analog tersubstitusi 2,3-difluoro, metil, bromo, dan cyano mempunyai indeks selektiviti ≥ 3. Kajian ini menonjolkan potensi kompleks argentum (I)-NHC sebagai sebatian calon antikanser, Kajian lanjutan perlu menyelidiki mekanisme kematian sel yang diinduksi oleh kompleks tersebut.

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ABSTRACT

Cancer remains a persistent global health challenge, necessitating innovative therapeutic agents. Metal-based drugs, particularly those with silver(I) ions, are promising due to their high reactivity and ability to form stable complexes with biological molecules, offering potential advantages over traditional treatments. Current chemotherapeutic drugs often cause severe toxicity and suffer drug resistance, which drives the development of new compounds with improved efficacy and selectivity. In this study, two new series of unsymmetrical benzyl-functionalized and their silver(I)-*N*-heterocyclic carbene (NHC) complexes were prepared. The benzimidazolium salts, **C2**-**C11**, were prepared via *N*-alkylation of 1-decylbenzimidazole with benzyl or substituted benzyl bromides, followed by *in situ* deprotonation with silver(I) oxide (Ag_2O) and a metathesis reaction with potassium hexafluorophosphate (KPF_6) to yield silver(I)-NHC complexes, **C12**-**C21**. These compounds were characterized using Fourier-transform infrared (FT-IR) spectroscopy and Fourier-transform nuclear magnetic resonance (FT-NMR) spectroscopy, liquid chromatography mass spectrometry (LC-MS), ultra-performance liquid chromatography mass spectrometry (UPLC-MS), and CHN elemental analysis. Their *in vitro* cytotoxicity was evaluated against human cervical cancer (HeLa) and breast cancer (MCF-7) cells, as well as normal skin fibroblast (Hs27) were evaluated *via* the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All compounds

exhibited substantial growth inhibition against the tested cell lines, outperforming the clinical anticancer drug, etoposide. Fluorination selectively enhanced cytotoxic effects on HeLa and Hs27 cells but had no enhanced cytotoxic effect against MCF-7 cells. *Para*-substituents (CH_3, Cl, Br, CN, NO_2) on the benzyl moiety modulated both potency and selectivity towards the tested cell lines, with methyl-substituted derivatives showing higher activity against MCF-7 cells and lower toxicity to Hs27 cells. The incorporation of silver(I) ions significantly enhanced the cytotoxic efficacy and selectivity of the silver(I)-NHC complexes, wherein 2,3-difluoro-, methyl-, bromo-, and cyano-substituted analogues had selectivity indices ≥ 3. This study highlights the potential of silver(I)-NHC complexes as anticancer lead compounds. Future studies should focus on the investigation of their cell death mechanisms.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cancer is a complex and multifaceted disease marked by uncontrolled cell proliferation and the potential to invade neighboring tissues, posing a significant global health challenge. The Global Cancer Observatory (2024) predicts a dramatic increase in cancer cases with approximately 32.6 million new cases projected worldwide by 2045. In Malaysia, cancer ranks the fourth leading cause of death, with cases anticipated to exceed 67,000 by 2030 (MySCan, 2018).

Currently, cancer treatment relies heavily on chemotherapy, radiotherapy, and surgery; however, many of these treatments are often accompanied by significant limitations. Chemotherapeutic drugs like cisplatin, discovered by Rosenberg and coworkers, paved the way for the development of metallodrugs (Figure 1.1) (Rosenberg, 1969). However, the clinical use of cisplatin and similar drugs, such as oxaliplatin, carboplatin, nedaplatin, and lobaplatin, is often limited by short- and long-term side effects, organ toxicity, allergic reactions, and the development of drug resistance over extended use (Oun et al., 2018). Consequently, researchers have focused on developing alternative metallodrugs that provide similar therapeutic benefits with reduced toxicity. This shift from cisplatin to a broader class of metallodrugs marks a significant advancement in the pursuit of more effective and targeted anticancer agents (Hannon, 2007).

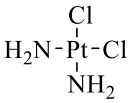


Figure 1. 1: Cisplatin, the first anticancer metallodrug (Rosenberg, 1969).

Metallodrugs, consisting of metal ions coordinated to organic ligands, have emerged as a prominent class of therapeutic agents used in treating cancers, infections, and inflammatory diseases. The incorporation of metals imparts unique properties to these agents, such as enhanced stability, reactivity, and biological activity. The metal ion often serves as the active component in these therapeutic agents, playing a crucial role in binding to biomolecules and disrupting cellular processes. Over the years, due to the diverse properties of metallodrugs, they have garnered considerable interests in inorganic medicinal chemistry due to their broad range of properties, such as anticancer, antimicrobial, and anti-inflammatory, as well as their potential preclinical and clinical applications in disease treatments (Ferraro & Merlino, 2022).

Metal complexes bearing the *N*-heterocyclic carbene (NHCs) ligands are among the many promising candidates in metallodrug design. Researchers have focused on this class of compounds because of their ease of preparation, unique structural and electronic properties. NHCs are cyclic carbenes that contain at least one nitrogen atom bound to a divalent carbon atom (Figure 1.2) (Benhamou et al., 2011). They exhibit strong σ-donor capabilities, acting as potent Lewis bases and excellent nucleophiles. These strong σ-donating properties lead to the formation of stable metal-ligand bonds, significantly enhancing the stability and solubility of these complexes. This in turn can increase

bioavailability of the metal-NHC complexes by facilitating the gradual release of metal ions *in vivo*, allowing for targeted delivery to cancer cells (Naderizadeh & Bayat, 2020).

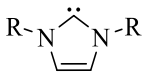


Figure 1. 2: A *N*-heterocyclic carbene (Benhamou et al., 2011).

A NHC precursor, benzimidazole is an aromatic heterocyclic compound consisting of a benzene ring fused to an imidazole moiety. It is an important scaffold in drug design and development (Figure 1.3) (Obot & Edouk, 2017). This structure allows for various chemical modifications, thus facilitating the design of drugs with a broad range of biological activities. Benzimidazole and its derivatives possess biological effects, such as anticancer, anti-inflammatory, antimicrobial, and antioxidant activities (Pathare & Bansode, 2021). For instance, bendamustine, a benzimidazole derivative, is a clinically approved anticancer agent (Figure 1.4) (Tageja & Nagi, 2010)

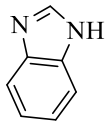
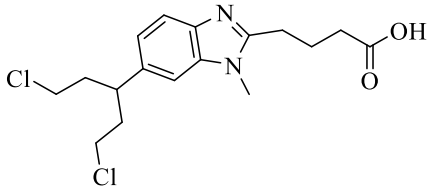


Figure 1. 3: Benzimidazole, a biologically important heterocycle (Obot & Edouk, 2017).



Bendamustine

Figure 1. 4: Bendamustine, a clinically approved anticancer drug (Tageja & Nagi, 2010).

Benzimidazolium-derived NHCs combine the beneficial properties of both the benzimidazole scaffold and the NHCs. The benzimidazole moiety is a bioactive scaffold, while the NHC enhances the stability and efficacy of metal complexes. The synergy between these two components enhances their therapeutic potential. Their widespread use in drug design and development highlights their crucial role in advancing medicinal chemistry, particularly in devising potent agents for various diseases. To date, many benzimidazolium-derived NHCs and their metal complexes have been extensively studied, revealing a broad spectrum of biological activities, including anticancer (Sahin-Bolukbası & Sahin, 2019), antimicrobial (Tutar et al., 2022), antioxidant (Geetha et al., 2020), antiparasitic (Touj et al., 2020), antiviral (Ustun et al., 2021) and anti-Alzheimer activities (Sandeli et al., 2022).

Silver and its derivatives have long been recognized for their medicinal properties, dating back to their use in wound healing in ancient times (Medici et al., 2019). Silver offers substantial advantages in drug formulations due to its strong antimicrobial properties against a wide spectrum of bacteria, fungi, protozoa, and viruses (Alexander, 2009). Its ability to enhance wound healing and inhibit biofilm formation makes it particularly useful in topical treatments. Furthermore, the potential of silver nanoparticles as carriers for other therapeutic agents could enhance drug delivery and efficacy (Gomes et al., 2021).

The exploration of the medicinal uses of silver sparked significant interest in the development of more complex silver-based compounds, including silver(I)-NHC complexes. Among these, silver(I)-NHC complexes derived from benzimidazolium salts

have garnered attention for their promising biological activities, particularly in cancer treatment. These complexes exhibit enhanced stability and reactivity compared to other types of silver complexes (Kalinowska-Lis et al., 2016). The enhanced anticancer activities can be attributed to their gradual and sustained release of silver ions, allowing them to maintain effective concentrations over extended periods, thereby enhancing their capacity to interact with targeted cancer cells (Tan et al., 2010).

Mono-silver(I)-NHC complexes, particularly those utilizing a simple benzimidazolium ligand, have shown promising anticancer properties against various cancer cells. For instance, Cevik-Yıldız and coworkers found that 1-allyl-substituted mono-silver(I)-NHC complex (1) exhibited significant inhibitory effects against human prostate cancer (DU-145) and human breast cancer (MCF-7; MDA-MB-231) cell lines, highlighting it as a potential anticancer agent (Figure 1.5) (Cevik-Yıldız et al., 2020). Building on this, bis-silver(I)-benzimidazolium NHC complexes featuring different alkyl groups (2) have shown enhanced cytotoxicity; for example, a propylated complex effectively targeted human erythromyeloblastoid leukemia (K-562), MCF-7, and colorectal adenocarcinoma (HCT116) cell lines (Figure 1.5) (Atif et al., 2020). Furthermore, multinuclear silver(I)-NHC complexes, including dinuclear (3) (Hussaini et al., 2017) and trinuclear variants (4) (Fatima et al., 2020), have exhibited substantial cytotoxic effects against a range of cancer cells (Figure 1.5). These multinuclear compounds often demonstrate superior activities compared to those of their mono- and bis-counterparts due to the presence of multiple silver centers, which enhance their interaction with biological targets.

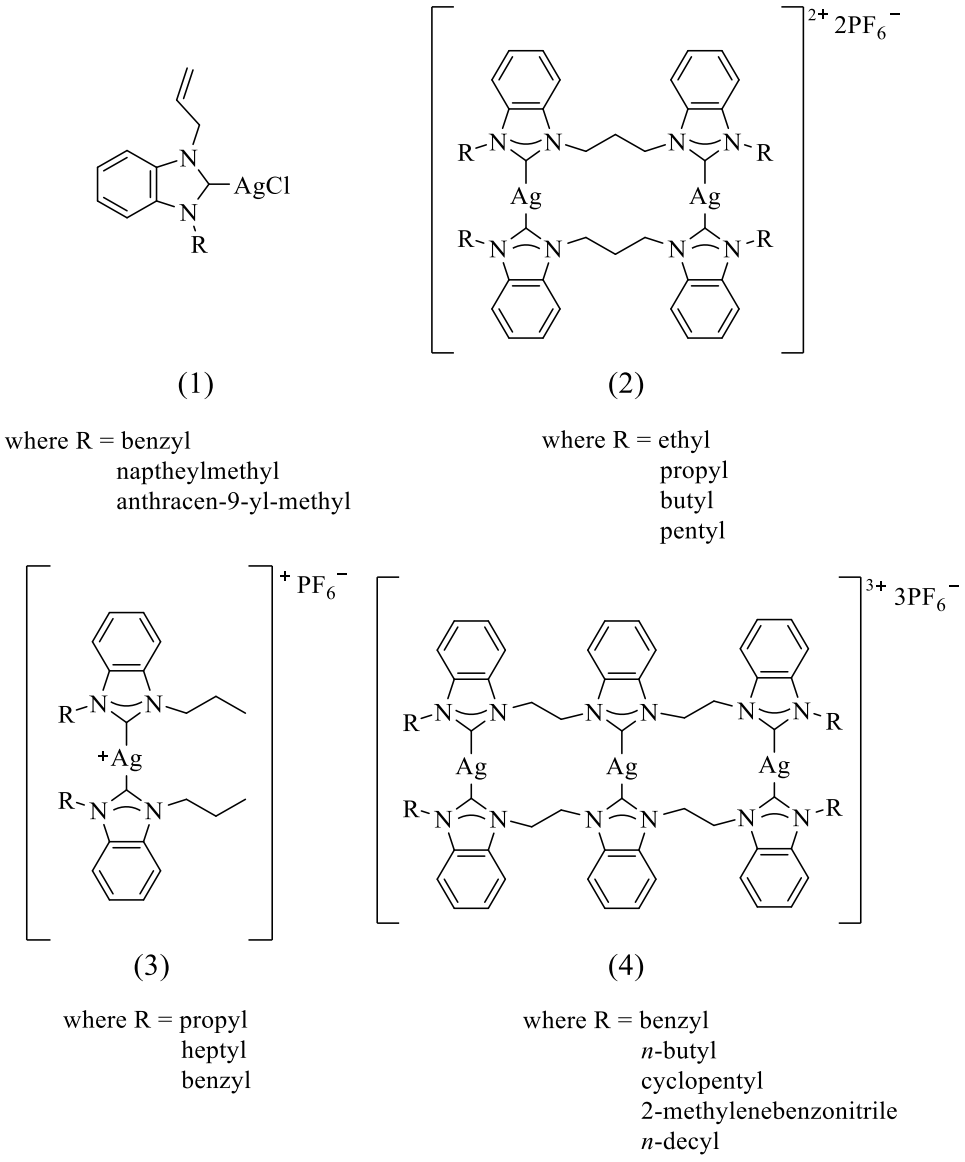
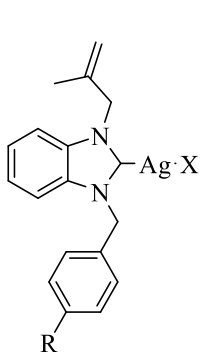


Figure 1. 5: Silver(I)-benzimidazolium NHC complexes as anticancer agents (Cevik-Yıldız et al., 2020; Fatima et al., 2020; Hussaini et al., 2017).

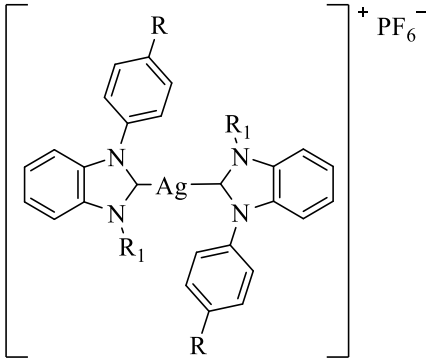
In addition to anticancer activity, silver(I)-benzimidazolium NHC complexes also exhibit notable antimicrobial properties. Mono-silver(I)-NHC complexes, such as those derived from 1-methallyl-benzimidazolium salts with *para*-substituted benzyl rings (5), demonstrate promising activity against *Candida albicans* (Figure 1.6) (Sahin et al., 2021a).

Furthermore, alkylated bis-silver(I)-NHC complexes (6) (Subramanya Prasad et al., 2017), bearing different electron-withdrawing substituents, showed significant potency against *Escherichia coli* and *Staphylococcus aureus*. Meanwhile, binuclear complexes (7)(Hussaini et al., 2021), characterized by two silver centers, enhanced antimicrobial efficacy against *E. coli* and *S. aureus* (Figure 1.6).



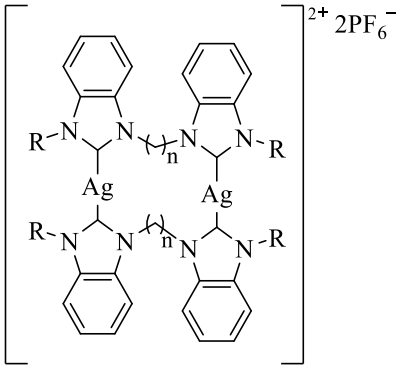
(5)

where X = Cl/Br
R = H
methyl
chloro
tert-butyl
methoxy



(6)

where R = NO_2/CN
R_1 = allyl/isopropyl/*sec*-butyl/isopentyl



(7)

where R = hexyl/heptyl/octyl/nonyl/decyl

Figure 1. 6: Silver(I)-benzimidazolium NHC complexes which exhibited antimicrobial activity (Sahin et al., 2021; Subramanya Prasad et al., 2017; Hussaini et al., 2021).

Understanding the structure-activity relationship (SAR) is essential for designing silver(I)-benzimidazolium NHC complexes, as it elucidates how different substituents impact their biological activities. Structural modifications such as adjusting alkyl chain lengths, introducing bulky substituents, and varying electronic properties of substituents significantly influence the efficacy and selectivity of these complexes. For example, incorporating electron-donating or electron-withdrawing groups alters the stability and reactivity of these complexes, increasing their therapeutic potential and enhancing their interaction with biological targets. Through strategic alterations of substituents, researchers can optimize the anticancer properties of these complexes. Increasing lipophilicity *via* longer alkyl chains can enhance cellular uptake, while bulky groups can improve binding affinity to specific receptors or enzymes (Spassov, 2024; Zhang et al., 2019).

The synthesis and characterization of benzyl-functionalized benzimidazolium salts and their corresponding silver(I)-NHC complexes in this study represent a significant advancement in the development of novel anticancer agents. NHCs are valued in coordination chemistry for their stability and versatility, and when coordinated with silver(I) ions, these complexes have demonstrated promising biological activities, particularly in inducing apoptosis in cancer cells. This research is impactful as it broadens the scope of silver(I)-NHC complexes by incorporating benzyl and substituted benzyl groups, potentially improving lipophilicity and cellular uptake, which are critical for enhancing anticancer effectiveness. Current cancer treatments, such as cisplatin, often face limitations like severe side effects and drug resistance. By exploring new structural variations, this study aims to identify compounds with improved selectivity and potency,

as evidenced by their in vitro cytotoxic effects on cancer cell lines. Thus, these synthesized compounds contribute to the ongoing effort to develop more effective and less toxic chemotherapeutic options, addressing a critical need in medicinal chemistry.

1.2 Problem Statement

The current clinically approved anticancer drugs face several critical issues, such as non-selectivity, development of drug resistance, and poor stability under physiological conditions, which lead to rapid degradation before reaching target cells. This urgency is underscored by alarming cancer statistics: The Global Cancer Observatory projects approximately 32.6 million new cancer cases worldwide by 2045, while in Malaysia, where cancer is the fourth leading cause of death, over 67,000 cases are anticipated by 2030. These figures highlight the critical need for innovative therapeutic approaches to combat this growing global health crisis. Advances in metallodrugs have prompted bioinorganic chemists to prepare a diverse range of metal-containing compounds. Among these, metal-NHC complexes show promising potential as anticancer agents. The NHC ligands can be easily functionalized, and the strong metal-NHC bond enhances drug stability, thus preventing premature dissociation, and hence prolong their bioavailability through the slow release of metal ions to target cells. Among the many transition metals, silver has reduced toxicity to humans. Silver(I)-NHC complexes have emerged as highly promising anticancer agents, with preclinical studies showing their potent cytotoxic effects, with some demonstrating selectivity for specific cancer cell lines.

The SAR is important in drug design, as it helps in understanding how a compound's chemical structure influences its biological activity and efficacy, thereby facilitating the optimization of both its efficacy and safety profiles of compound against

cancer cells. Despite significant progresses in the design and development of cytotoxic silver(I)-NHC complexes, a literature search revealed a lack of a comprehensive SAR data on silver(I)-benzimidazolium NHC complexes. The cytotoxicity of these complexes is significantly influenced by the lipophilicity, steric, and electronic properties of the substituents at N1 and N3 atoms in the benzimidazolium moiety. In this study, the silver(I)-benzimidazolium NHC complexes were strategically modified to optimize their anticancer efficacy. The complexes in Series I were functionalized with different fluorinated benzyl moieties and a decyl chain to explore how fluorination affects the anticancer activity. Additionally, complexes in Series II were designed with *para*-substituted benzyl moieties and a decyl chain to study the substituent effects on cytotoxicity.

To assess the therapeutic potential and safety of modified silver(I)-benzimidazolium-NHC complexes, *in vitro* cytotoxicity effects against HeLa, MCF-7 and Hs27 were evaluated. The MCF-7 and HeLa cells were selected as well-established models for prevalent and aggressive cancers, enabling evaluation of the efficacy of the compounds against clinically significant targets. Meanwhile, Hs27 served as a control to determine selectivity, ensuring the compounds target cancer cells while sparing healthy ones. This strategic choice of cell lines supports a comprehensive analysis of both potency and safety, advancing the development of effective and tolerable anticancer treatments.

1.3 Objectives

The objectives of this research are:

1.3.1 To synthesize and characterize two new series of substituted benzyl-functionalized benzimidazolium salts and their silver(I)-NHC complexes.

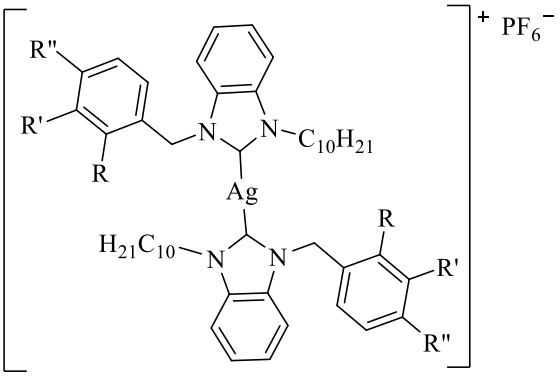
1.3.2 To evaluate the *in vitro* cytotoxic effects of the salts and their silver(I)-NHC complexes against Hs27, HeLa, and MCF-7 using the MTT assay.

1.3.3 To determine the impact of substituents in the benzyl moiety on cytotoxicity of benzimidazolium salts and their silver(I)-NHC complexes against the tested cell lines.

1.4. Scope of study

In this research, two new series of substituted benzyl-functionalized benzimidazolium salts and their silver(I)-NHC complexes were prepared. These complexes varied in terms of the number of fluorine atoms, fluorination patterns, and *para*-substituents in the benzyl moiety. The salts were synthesized through *N*-alkylation of decylbenzimidazole with benzyl bromides or substituted benzyl bromides. Reactions of the salts with silver(I) oxide (Ag_2O) yielded the silver(I)-NHC complexes (Figure 1.7). The synthesized compounds were characterized using Fourier-transform infrared (FT-IR) spectroscopy, Fourier-transform nuclear magnetic resonance (FT-NMR) spectroscopy, liquid chromatography mass spectrometry (LC-MS), ultra-performance liquid chromatography mass spectrometry (UPLC-MS), and carbon, hydrogen, nitrogen (CHN) analysis. The *in vitro* cytotoxicity of the salts and their silver(I)-NHC complexes against HeLa, MCF-7, and Hs27 cells was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The effects of the number and position of fluorine atoms, as well as the *para*-substituents, on cytotoxicity were discussed. Cellular toxicity of silver-NHC complexes is a complex process that may involve multiple pathways. Unfortunately, due to time and financial constraints, the investigation into the cellular

uptake and detailed mechanisms of action of the complexes in this study could not be conducted.



Series I:

where R = R' = R" = H

R = F; R' = R" = H

R = R' = H; R" = F

R = R' = F; R" = H

R = R' = H; R" = CF_3

Series II:

where R = R' = H; R" = CH_3/Cl/Br/CN/NO_2

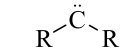
Figure 1. 7: Silver(I)-benzimidazolium NHC complexes prepared in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Carbenes and *N*-heterocyclic carbenes (NHCs)

Carbenes are neutral compounds characterized by a divalent carbon atom with a six-electrons valence shell and a formal charge of zero (Figure 2.1). The incomplete octet and coordinative unsaturation make carbenes highly reactive (Tuna Subasi, 2022).



R= H/alkyl/aryl

Figure 2. 1: General structure of carbene (Tuna Subasi, 2022).

Each carbene carbon has three sp^2 hybridized orbitals: two form covalent bonds, while the lone pair occupies either an sp^2 hybridized orbital or the *p* orbital (Sakander et al., 2023). The electron configuration determines whether a carbene is a singlet or triplet. Singlet carbenes have opposite-spin electrons in the sp^2 orbital, whilst triplet carbenes have parallel-spin electrons, each in the sp^2 and *p* orbitals (Figure 2.2) (Tuna Subasi, 2022).

Singlet Carbene Triplet Carbene

Figure 2. 2: Electron configuration of singlet and triplet carbenes (Tuna Subasi, 2022).

The electron spin states in carbenes are influenced by their substituents. Electron-withdrawing groups tend to stabilize singlet carbenes by increasing the energy gap between the σ- and π-orbitals, encouraging electron occupation in the σ-orbital. Conversely, triplet carbenes are stabilized by electron-donating groups that reduce the energy gap, making both σ- and π-orbitals more accessible (Manna et al., 2022.; Tomioka, 1997).

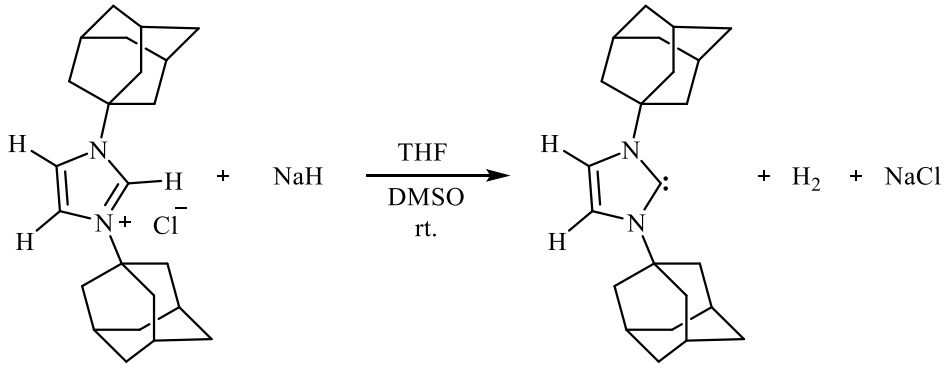
The two important types of carbenes are Fischer and Schrock carbenes (Figure 2.3). The major differences between Fischer and Schrock carbenes are their spin states, and the nature of their interactions with metals. The more stable and electrophilic Fischer carbenes, discovered by Ernst Otto Fischer in 1964, are singlet carbenes that use their pair of electrons to form covalent bonds with metals through σ-donation and π-back-donation, resulting in electron density polarizing toward the metal. These interactions make Fischer carbenes versatile intermediate in chemical synthesis (Fischer & Maasböl, 1964.; Santamaría & Aguilar, 2016). On the other hand, Schrock carbenes which are highly reactive and nucleophilic were discovered by Richard Royce Schrock in 1974. These triplet carbenes have two unpaired electrons that polarize toward the carbene carbon, forming covalent bonds where the electron density shifts away from the metal (Schrock, 1974; Santamaría & Aguilar, 2016).

Fischer Carbene (a)

Schrock Carbene (b)

Figure 2. 3: General structure of (a) Fischer carbene, and (b) Schrock carbene (Santamaría & Aguilar, 2016).

NHCs are cyclic singlet carbenes featuring two π-donating nitrogen atoms. In 1991, Arduengo and coworkers prepared and reported the first isolable NHC, namely 1,3-di(1-adamantyl)imidazole-2-ylidine, via *in situ* deprotonation of 1,3-di(1-adamantyl)imidazolium chloride (Scheme 2.1) (Arduengo et al., 1991). This carbene was very stable at room temperature, which spurred research interest in NHCs.



Scheme 2. 1: The first free NHC, 1,3-di(1-adamantyl)imidazole-2-ylidine (Arduengo et al., 1991).

Since the discovery and isolation of the first free NHC by Arduengo and coworkers, NHCs have become widely recognized for their versatility in coordination chemistry. The main advantages of NHCs include ease of preparation, low toxicity, and the ability to form strong bonds with transition metals (Droge & Glorius, 2010; Hopkinson et al., 2014). The reactivity and interactions of NHCs with metal centers can be influenced by adjusting their *N*-substituents and ring size, which modulate both their electronic and

steric properties. The tunability of NHCs makes them highly effective ligands in metal catalysis (Danopoulos et al., 2019; Froese et al., 2017).

2.2 Benzimidazolium salts as *N*-heterocyclic-carbenes precursors

NHCs have become widely recognized for their versatility in coordination chemistry due to their ability to form strong bonds with metal centers. NHCs are typically synthesized from azolium salts, such as imidazolium and benzimidazolium salts, which serve as precursors for these ligands (Furstner et al., 2006). Among these, benzimidazolium-based NHC precursors have gained particular interest due to their stability and effectiveness in forming metal complexes, especially with transition metals like silver. Several synthetic routes have been developed for the preparation of these benzimidazolium salts, making them ideal candidates for further exploration in coordination chemistry and potential anticancer applications (Benhamou et al., 2011).

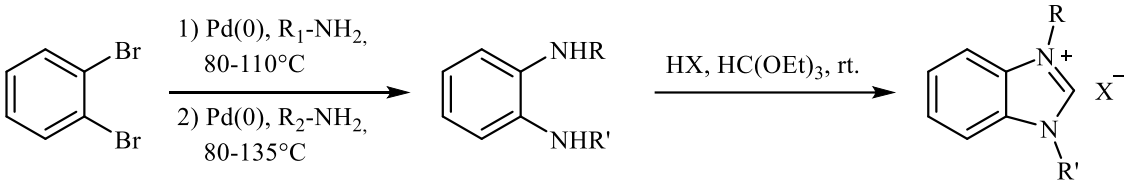
2.2.1 Preparation of benzimidazolium salts

The preparation of benzimidazolium salts is typically achieved through methods such as amination and cyclization or oxidation of *ortho*-phenylenediamine. Alternatively, nucleophilic substitution at the azole heterocycles offers a direct route to introduce desired substituents (Benhamou et al., 2011).

2.2.1.(a) Amination and cyclization route

The synthesis of benzimidazolium salts can be effectively achieved through a tandem Buchwald-Hartwig amination followed by cyclization with triethyl orthoformate

(Scheme 2.2) (Rivas et al., 2001). The Buchwald-Hartwig amination is a chemical reaction that forms carbon-nitrogen bonds by coupling an aryl halide with an amine, catalyzed by a palladium complex. This method begins with the palladium-catalyzed amination of 1,2-dibromobenzene, allowing for the introduction of various amines, including chiral amines, to create *N*-substituted intermediates. The subsequent cyclization step is performed using triethyl orthoformate, which facilitates the formation of the benzimidazolium salt (Rivas et al., 2001).

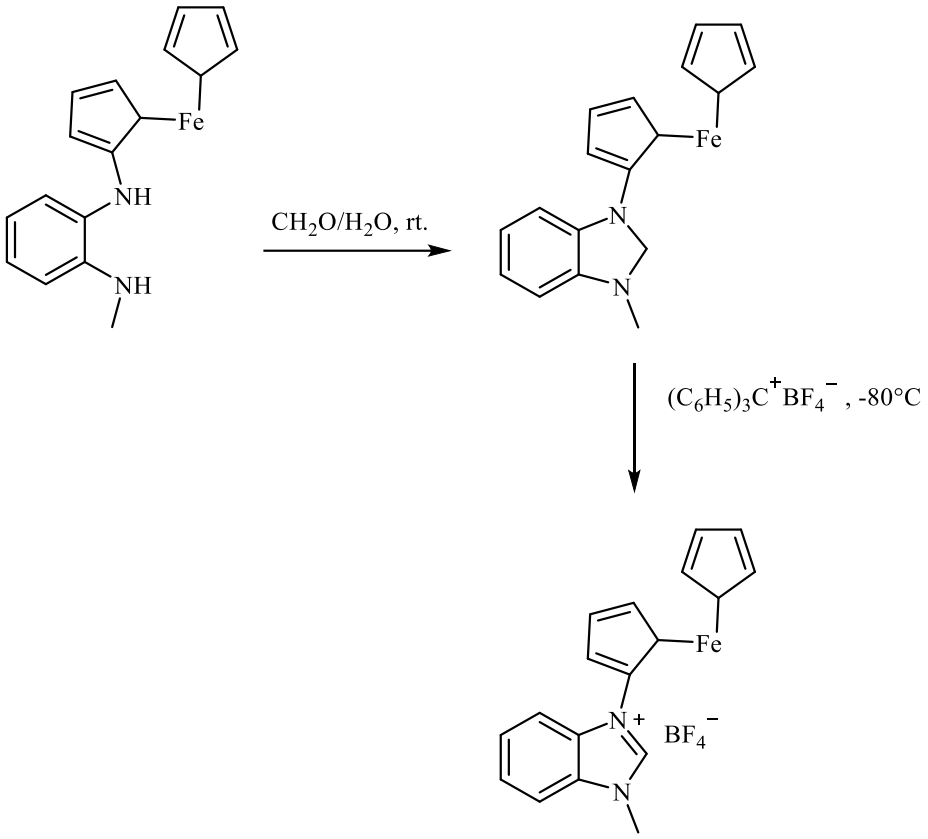


where X = Cl/ClO_4
R = R' = 1-phenylethyl
R = 1-phenylethyl, R' = 1-phenyl
R = 1-phenyl-2-methoxyethyl, R' = 1-phenyl

Scheme 2. 2: Synthesis of the benzimidazolium salts *via* tandem Buchwald-Hartwig amination and cyclization reaction (Rivas et al., 2001).

2.2.1(b) Oxidation of *ortho*-phenylenediamine

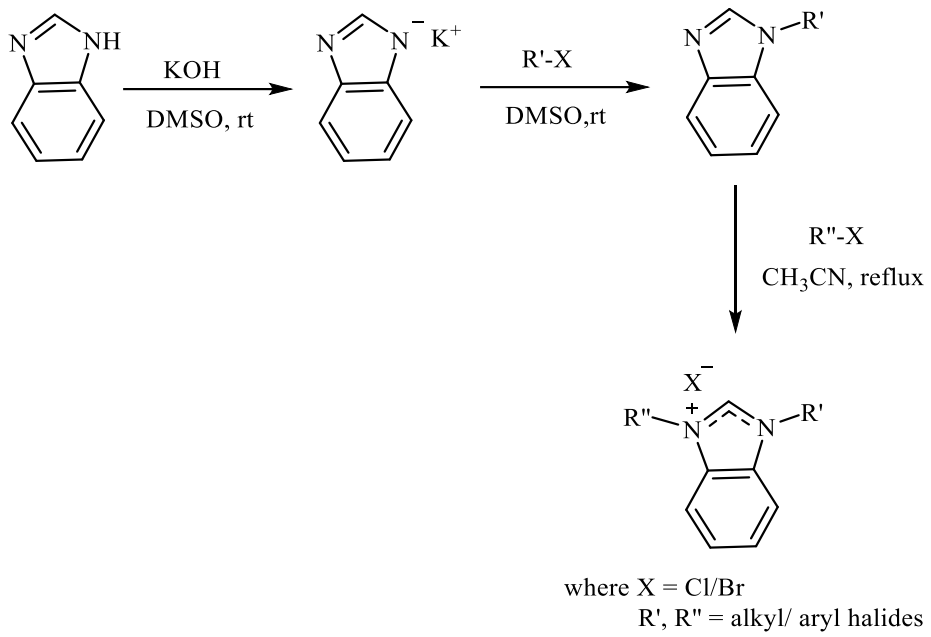
Bildstein and coworkers (1999) synthesized benzimidazolium salts by first coupling a metallocenyl group to *ortho*-phenylenediamine precursor. This was followed by ring closure and oxidation of the *ortho*-phenylenediamine. They reacted *N*-ferrocenyl-*N*-methyl-1,2-diaminobenzene with aqueous formaldehyde to yield 1-ferrocenyl-3-methyl-benzimidazoline. This intermediate was then oxidized using trityl tetrafluoroborate, resulting in the formation of benzimidazolium tetrafluoroborate.



Scheme 2. 3: Synthesis of the benzimidazolium salts *via* oxidation of *ortho*-phenylenediamine (Bildstein et al., 1999).

2.2.1(c) Nucleophilic substitution at the azole heterocycles

Nucleophilic substitution by an alkyl or aryl moiety at the nitrogen atoms in azole heterocycles is a common method for synthesizing symmetrical or unsymmetrical *N*, *N*'-dialkylated benzimidazolium salts in high yields (Scheme 2.4) (Starikova et al., 2003).

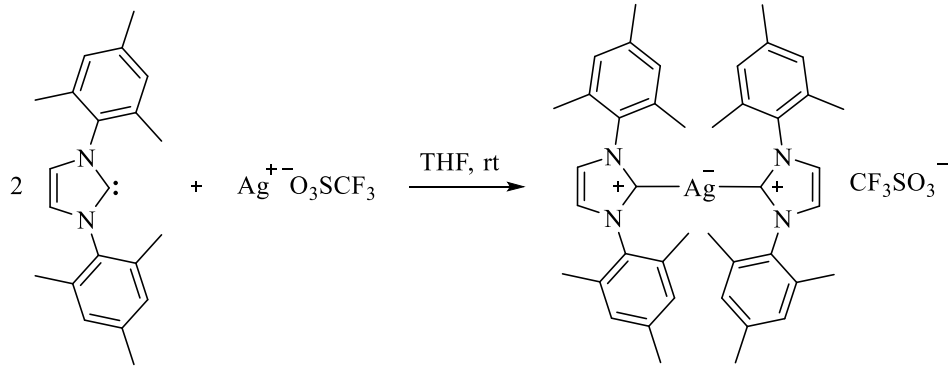


Scheme 2. 4: Synthesis of symmetrical and unsymmetrical benzimidazolium salts (Starikova et al., 2003).

2.3 Silver(I)-*N*-heterocyclic carbenes complexes derived from benzimidazolium salts

NHCs are important ligands in transition metal complexes because of their structural and electronic versatility. Compared to metal complexes with alternative coordination ligands such as phosphines, amines, or carbonyls, metal-NHC complexes exhibit enhanced lipophilicity, kinetic stability, and electronic stability (Kankala et al., 2018). The therapeutic potential of silver has been recognized for centuries, leading to its extensive use in medicinal chemistry. This historical application marks a significant breakthrough in the advancement of silver-based pharmaceuticals. The first reported silver(I)-NHC complex, synthesised by Arduengo and coworkers (Arduengo et al., 1993),

was a homoleptic bis(carbene) adduct of silver(I), prepared from 1,3-dimesitylimidazol-2-ylidene and silver(I) triflate (Scheme 2.5).



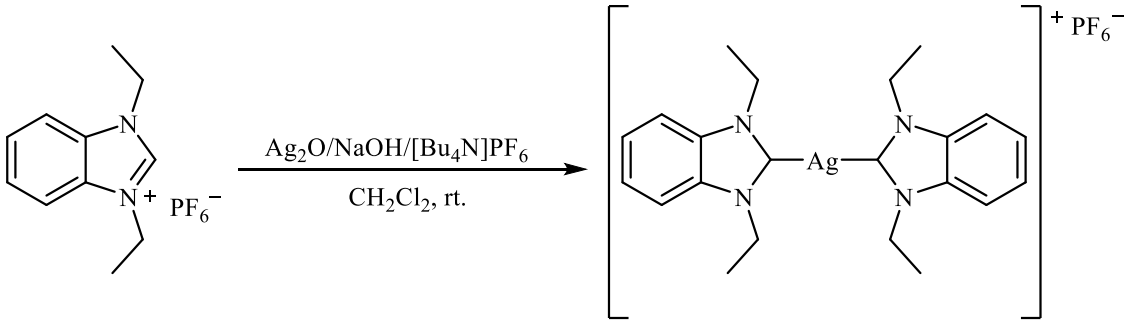
Scheme 2. 5: The first silver(I)-NHC complex synthesized from 1,3-dimesitylimidazol-2-ylidene and silver(I) triflate (Arduengo et al., 1993).

The incorporation of benzimidazole-derived NHCs into silver(I) complexes not only leverages the antimicrobial properties of silver but also introduces the potential for enhanced anticancer activity (Raju et al., 2022; Ronga et al., 2023). The structural versatility of benzimidazolium salts facilitates fine-tuning of electronic and steric properties, enabling the design of silver(I)-NHC complexes with optimized biological performance.

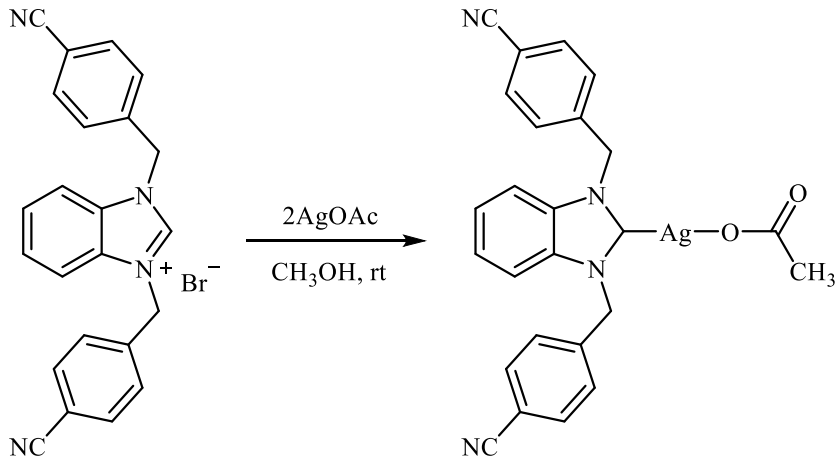
Typically, the synthesis of silver(I)-NHC complexes involves the *in situ* deprotonation of benzimidazolium salts using metal salts such as Ag_2O, silver acetate ($Ag_2C_2H_3O_2$) and silver carbonate (Ag_2CO_3). This method allows direct reaction of the benzimidazolium salt with the silver salt, facilitating the formation of coordination complexes. The resultant NHC ligands coordinate to the silver ion *via* a divalent carbon

center, enhancing the stability of the complex through strong σ-donation from the ligand and potential π-back donation from the metal (Bourissou et al., 2000).

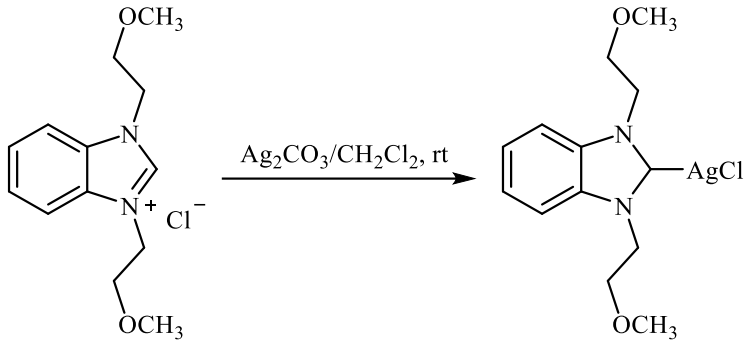
Wang and Lin (1998) pioneered the synthesis of silver(I)-benzimidazolium NHC complexes using Ag_2O as the silver source, resulted in high yields (89%) (1) (Scheme 2.6). This method is more effective, yielding higher amounts than those obtained using $Ag_2C_2H_3O_2$ (2) (79%) and Ag_2CO_3 (3) (87%) (Scheme 2.6) (Gok et al., 2013; S. Patil et al., 2010). Ag_2O, consistently providing the highest yields, was particularly valuable for synthesizing silver(I)-benzimidazolium NHC complexes.



(1)



(2)



(3)

Scheme 2. 6: *In situ* deprotonation of azolium salts by several silver salts to form silver(I)-NHC complexes (Gok et al., 2013; Patil et al., 2010; Wang & Lin, 1998).

2.4 Biological activities of silver(I)-*N*-heterocyclic carbene complexes derived from benzimidazolium salts

Silver has historically been recognized for its antimicrobial properties, with ancient civilizations, such as the Greeks and Romans, utilizing silver vessels to store liquids and inhibit bacterial growth (Melaiye & Youngs, 2005). Its application in medicine continued through the ages, gaining prominence in 1881 when Carl Siegmund Franz Crede introduced a 2% silver nitrate solution to prevent ophthalmia neonatorum, a form of conjunctivitis caused by bacterial infection in newborns (Crede, 2019). This intervention highlighted silver's therapeutic potential. However, after World War II, the use of silver-based treatments declined as antibiotics like penicillin and sulfonamides became more widespread (Klasen, 2000). The resurgence of antibiotic-resistant bacterial strains, such as *Pseudomonas aeruginosa* and *S. aureus*, revived interest in silver

compounds, leading to the development of silver sulfadiazine, an antimicrobial agent used in burn wound care (Fox, 1968).

Although silver is not a naturally occurring element in the human body, its derivatives are valued for their low toxicity and therapeutic potentials, including anticancer activity. A significant limitation of silver-based drugs is the rapid release of silver ions, which can diminish their therapeutic efficacy. This challenge can be addressed by incorporating NHCs as ligands. NHCs exhibit a strong affinity for various metals, including palladium, platinum, and gold, which enhances their versatility in catalytic and therapeutic applications. Specifically, their strong affinity for silver enables a steady and continuous release of silver ions into cell membranes and improving of the drug (Liu & Gust, 2016). Particularly, silver(I)-NHC complexes derived from benzimidazolium salts have been demonstrated a broad range of biological activities, such as anticancer, antimicrobial, and enzyme inhibition, which are attributed to their lipophilicity and kinetic stability (Lasmari et al., 2021).

2.4.1 Anticancer activity of silver(I)-*N*-heterocyclic carbene complexes derived from benzimidazolium salts

Patil and coworkers (2011) reported that the benzyl-substituted mono-silver(I)-benzimidazolium based NHC complexes (4) exhibited stronger anticancer activity (IC_{50} = 10.80 μM) against cancerous renal cell line (Caki-1), compared to the *para*-cyanobenzyl analogue (IC_{50} = 24.20 μM) (Figure 2.4), suggesting that electron-withdrawing groups reduce potency. However, Yasar and coworkers observed that the anticancer activities increased with stronger electron-donating ability NHC-bearing silver(I) complexes (5)

(Figure 2.4) (Yasar et al., 2018). The most potent complexes, featuring the 2,3,4,5,6-pentamethyl group with the strong electron-donating ability, exhibited the highest activity against HeLa and human colorectal adenocarcinoma (HT29) cell lines (IC_{50} = 5.99 and 13.87 μM, respectively). These findings of a direct correlation between electron-donating ability and increased anticancer efficacy were supported by Sahin-Bolukbası and coworkers (Sahin et al., 2019; Sahin-Bolukbaset al., 2019; Sahin-Bolukbas & Sahin, 2019). The 1-allyl-substituted silver(I)-benzimidazolium NHC complexes (6), containing 2,3,4,5,6-pentamethyl substituents, exhibited greatest potency against DU-145, MCF-7, and MDA-MB-231 cell lines (IC_{50} < 1.21 μM) (Figure 2.4).

Other studies highlighted the steric influence on cytotoxicity. Aktas and coworkers (2017) studied the anticancer effect of silver(I) complexes derived from morpholinoethyl-substituted benzimidazolium salts (**7**) against MCF-7 cell lines (Figure 2.4). Their findings revealed that the anticancer activity enhanced as more sterically bulky *N*-substituents were incorporated, wherein complexes bearing 2,3,5,6-tetramethylbenzyl groups showed highest efficacy against MCF-7 cell lines (IC_{50} = 6.56 μM). The planar geometry of the 2,3,5,6-tetramethylbenzyl groups allows for a larger surface area for cellular interaction, enhancing their ability to target cancer cells. Cevik-Yıldız and coworkers found that 1-allyl mono-silver(I)-benzimidazolium NHC complexes (8) with the bulkiest anthracen-9-yl-methyl groups was the most active against DU-145, MCF-7, and MDA-MB-231 cell lines (IC_{50} < 2.95 μM) (Figure 2.4) (Cevik-Yıldız et al., 2020). This highlighted the increased in steric hindrances enhances the anticancer potency. However, Slimani and coworkers reported an opposing perspective, highlighting hat less sterically hindered *N*-isobutyl-substituted benzimidazolium silver(I)-NHC complexes, featuring methoxyethyl