DNA BINDING, NUCLEOLYTIC PROPERTIES, AND TOPOISOMERASE I INHIBITION OF TERNARY COPPER(II) COMPLEXES OF 1, 10-PHENANTHROLINE AND CHIRAL AMINO ACID

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ABSTRACT

DNA BINDING, NUCLEOLYTIC PROPERTIES, AND TOPOISOMERASE I INHIBITION OF TERNARY COPPER(II) COMPLEXES OF 1, 10-PHENANTHROLINE AND CHIRAL AMINO ACID

Nucleolytic and DNA binding properties of three pairs of ternary copper(II) complexes with 1,10-phenanthroline (phen) as main ligand and chiral amino acid (aa) as variable subsidiary ligand were studied by using agarose gel electrolysis, fluorescence quenching assay, restriction enzyme inhibition and topoisomerase I inhibition test. These complexes can be designated as $[Cu(phen)(aa)(H_2O)]X$ where X is a chloride or nitrate. The amino acids used were L-alanine, D-alanine, L-threonine and D-threonine. The experiments were divided into two main parts, viz. nucleolytic study and DNA interaction study, and the main objective of these experiments was to find out the factors involved in affecting the nucleolytic and DNA binding properties of copper(II) complexes.

The type of subsidiary ligand was shown to influence the DNA cleavage efficiency. Other factors, namely concentration of $[Cu(phen)(aa)(H_2O)]X$, incubation time, type of buffer, buffer pH, type of ligand, and exogenous agents (oxidizing or reducing) were important in determining the nucleolytic efficiency

of the copper(II) complexes in the *in vitro* studies. Moreover, the DNA cleavage mechanism in the presence or absence of exogenous agent was found to be different. However, there was no or insignificant effect on the nucleolytic properties of [Cu(phen)(aa)(H_2O)]X when the amino acid was changed from the L-form to the D-form.

In the DNA binding study, binding constants of the copper(II) complexes with various type of DNA were evaluated by ethidium bromide or thiazole orange quenching assay. The DNA chosen for this study were calf-thymus DNA (CT-DNA), ds$(AT)_6$, ds$(CG)_6$, G-quadruplex (telo21) and its corresponding duplex. Analysis of their binding constants revealed the effect of the type of amino acid and chirality of the amino acid. The copper(II) complexes show varying degree of binding affinity. Investigation into the DNA binding selectivity or specificity of the copper(II) complexes through the use of a series of restriction enzymes found that these complexes inhibit only some of the restriction enzymes, suggesting DNA binding selectivity. It was also found that the chirality of the amino acid could affect the binding selectivity.

With the aim of exploring new G-quadruplex DNA binder, the interaction of the copper(II) complexes with G-quadruplex annealed from human telomeric DNA, d[$G_3(T_2AG_3)_3$] (telo21) was investigated. Among the results, copper(II) complexes with chiral L-amino acids were shown to have slightly higher selectivity towards 22-nucleobase G-quadruplex than those with D-amino acids.

Lastly, most of the copper(II) complexes studied in this research work were found to have the ability to inhibit the function of human DNA topoisomerase I (topo I) in relaxing the supercoiled plasmid DNA, pBR322. The results suggested that the degree of inhibition of the topo I depended on the concentration of the copper(II) complexes. For some of the copper(II) complexes, the efficacy of the topo I inhibition was affected by the sequence of mixing the three components, suggesting that two types of inhibition mechanism. Inhibition could result from (i) copper(II) complexes binding to the DNA and blocking the topo I from functioning or (ii) the copper(II) complexes binding to the topo I and thereby affecting its function.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____ Date: _____

JAMES CHONG KOK UEI

APPROVAL SHEET

This dissertation entitled **"DNA BINDING, NUCLEOLYTIC PROPERTIES, AND TOPOISOMERASE I INHIBITION OF TERNARY COPPER(II) COMPLEXES OF 1, 10-PHENANTHROLINE AND CHIRAL AMINO ACID"** was prepared by JAMES CHONG KOK UEI and submitted as partial fulfillment of the requirements for the degree of Master in Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **JAMES CHONG KOK UEI** (ID No: **09UEM02357**) has completed this final year project entitled "**DNA BINDING, NUCLEOLYTIC PROPERTIES, AND TOPOISOMERASE I INHIBITION OF TERNARY COPPER(II) COMPLEXES OF 1, 10-PHENANTHROLINE AND CHIRAL AMINO ACID**" supervised by Assoc. Prof. Dr. Ng Chew Hee (Supervisor) and Assoc. Prof. Dr. Alan Ong Han Kiat (Co-Spervisor) from the Department of Bioscience, Faculty of Engineering and Science, Universiti Tunku Abdul Rahman.

I hereby give permission to my supervisors to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisors.

LIST OF ABBREVIATIONS

aa	Amino Acid
AA	Ascorbic Acid
Ala	Alanine
CT DNA	Calf Thymus Deoxyribonucleic Acid
$CuCl_2$	Copper(II) Chloride
H_2O_2	Hydrogen Peroxide
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HN	Hepes-NaCl Buffer
Phen	1,10-Phenanthroline
PN	Phosphate-NaCl Buffer
Pro	Proline
RE	Restriction Enzymes
TAE	Tris-acetate-EDTA Buffer
Threo	Threonine
TN	Tris-Nacl Buffer
Topo	Topoisomerase
Tris	Tris(hydroxylmethyl)amino-methane

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Many diseases are found to be related to our genes in DNA. Deoxyribonucleic acid, or commonly known as DNA, is the nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses.

Generally, the main role of DNA molecules is long-term storage of information. This information is mainly stored in the cell nucleus, but a small amount of DNA can also be found in the mitochondria (Bruce *et al.*, 2002). DNA is often compared to a set of blueprints, a recipe, or a code. It is involved several vital processes, such as gene expression, gene transcription, mutagenesis, as well as carcinogenesis (Miller EC and Miller JA, 1983). Moreover, DNA also contains the instructions needed to construct other components of the cells, such as proteins and RNA molecules (Saenger, 1984; Boddy and Russell, 2001). Since these DNA codes are important for several processes in all organisms, the existence of a set of regulatory system is relatively important. In addition, the failure of this regulatory system may lead to several downstream effects, such as mutation, uncontrolled cell growth, induction of tumor and cancer.

Substantial progress has been made during the past few decades to develop metal-based small molecules as DNA foot-printing, diagnostic, as well as therapeutic agents that are able to bind or cleave DNA under certain physiological conditions (Meurier, 1992; Sigman *et al.,* 1993; Sigman *et al.,* 1993; Pratviel *et al.,* 1995; Armitage, 1998; Pratviel *et al.,* 1998; McMillin and McNett, 1998; Erkkila *et al.*, 1999; Jamieson and Lippard, 1999; Metcalfe and Thomas, 2003; Chifotides and Dunbar, 2005). Metal complexes, in this context, with tunable coordination environment and versatile physicochemical properties, offer a wide scope for designing and developing highly sensitive pharmaceutical agents for medicinal applications (Umezawa, 1976; Lippard and Berg, 1994).

The design of metal complexes that target desired DNA sequences, for example, quadruplex, AT-rich and CG-rich sequence, has been one of the major challenges in the field of molecular recognition. These compounds have a wide range of potential applications, which are dependent on their ability to bind to the DNA (Barton, 1986; Friedman *et al.,* 1990; Arkin *et al.*, 1996; Nordén *et al.*, 1996; Liu *et al.*, 2000; Foley *et al.*, 2001; Zhou *et al.*, 2001; Mei *et al.*, 2003). Complexes could be potential candidates as synthetic restriction enzymes, new drugs, DNA foot-printing agents, stereoselective probes of nucleic acid structure and so on. It is very important to have a detailed understanding on the complex-DNA interaction in order to identify the potential application of the metal complexes.

Generally, the pharmacological activity and cleavage ability of the transition metal complexes have been found to be largely dependent on the nature of metal ion, the subsidiary ligands (Deepalatha *et al.*, 2005), size, as well as the stereochemical properties of the metal complexes (Vijayalakshimi *et al.*, 2005). All different types of interaction or binding will bring different downstream effects (Liu *et al.*, 2002). A complex can bind to DNA by means of several types of interactions, viz. hydrogen bond, electrostatic interaction, Van der Waals forces and so on. All these interactions showed different binding strength and selectivity. Udenfriend and Zaltzman (1961) suggested that the qualitative and quantitative analysis of nucleic acids as the material base of genetic inheritance is becoming more and more important nowadays. In quantitative analysis, their natural fluorescence intensity is however so weak that fluorescence spectrum has scarcely been used for studying biological properties. To overcome this problem, usually some fluorescent probes, for example ethidium bromide, thiazole orange, acridine orange, phosphin 3R and nile blue, have been employed for the investigation. Under certain conditions, fluorescence metal complexes can also be used for investigation purpose.

Several studies showed that transition metal ions, in particularly, copper ions, are able to influence the interaction between DNA and drugs, and it will result in conformational changes of the DNA structure (Hackl *et al.*, 2005). Ishida *et al.* (2002) found that copper is a competitive inhibitor of cisplatin uptake. Copper has been recognized as an essential trace metal for living organisms since

the late 1930s. Copper is essential in all plants and animals. In animals, including humans, it is found widely in tissues, with different concentration in liver, muscle, and bone. Copper is found in a variety of enzymes. It functions as a co-factor in various enzymes and in copper-based pigments, such as copper centers of cytochrome c oxidase, the Cu-Zn containing enzyme superoxide dismutase, and is the central metal in the oxygen carrying pigment hemocyanin. In addition to its enzymatic roles, copper is also used for biological electron transport (Linder, 2001).

In recent years, there has been continuous interest in determining the mode and extent of binding of metal complexes with DNA (Raja *et al.*, 2005). Such information is important to understand the cleavage pattern. Another purpose is to develop cleaving agents for probing nucleic acid structure and for other applications. It has been suggested that metal complexes interact with DNA by several types of binding, which can be classified as covalent and non-covalent binding. Several studies listed these interactions as having site-specific mechanism (Chevion, 1988), intercalative, electrostatic, minor- or major-groove (surface) binding, and inner-sphere coordination (Henderson and Dougherty, 1992; Sessler *et al.*, 1994; Harada *et al.*, 1996; Sternberg and Dolphin, 1998; Ali and Van Lier, 1999; Henderson *et al.*, 2000; Ackroyd *et al.*, 2001; Boerner and Zaleski, 2005).

Ever since the use of Cisplatin as antitumor drug, the research on the potential use of metal complexes as pharmaceuticals has gain momentum. The discovery that diamminedichloridoplatinum(II) ($Pt(NH_3)_2Cl_2$), or known as Cisplatin, promotes cancer cell death by binding to DNA *via* chloride ligand exchange (Zhang and Lippard, 2003). The steadily emerging understanding of the role of metal ions in restriction endonuclease (Cowan, 2004), hydrolase and phosphatase activity have also ignited a firestorm of work to examine the interaction of metal complexes with both DNA (Cowan, 2001) and RNA (Morrow and Iranzo, 2004). However, the development of these metallo-drugs is hindered by some major problems such as serious toxicity, poor solubility in water, side effects and resistance (Galanski *et al.*, 2005; Brabec and Nováková, 2006). These unresolved problems in platinum-based anticancer therapy have stimulated efforts to search for other novel non-platinum-based metal species as cytostatic agents (Ott and Gust, 2007).

Among the metal complexes so far investigated, those complexes of polypyridyl bases have attracted great attention by virtue of its binding propensity to nucleic acid under the physiological condition (Burger, 1998; Zeglis *et al.*, 2007; Janaratne *et al.*, 2007; Delaney *et al.*, 2002; Erkkila *et al.*, 1999). 1,10-Phenanthroline (abbreviated as phen) is one of the earliest and most extensively studied N-heterocyclic chelating agents. Because of the potential application as nonradioactive nucleic acid probes and DNA cleaving agents, the complexes of 1,10-phenanthroline and other polypyridyls with transition metals have been

investigated by many researchers (Sigman, *et al.*, 1993). Strong binding affinity of the copper bis phen complex and the redox behavior of the copper center played an important role in inducing oxidative DNA cleavage (Chetana *et al.*, 2009). In addition, recently there are a few studies extensively exploring the transition metal-based chemistry toward cleavage of DNA under the physiological condition by oxidative as well as photochemical means involving charge transfer or d–d band excitation (Pogozelski and Tullius, 1998; Dhar *et al.*, 2003; Dhar and Chakravarty, 2003; Chakravarty, 2006; Reddy *et al.*, 2004, Roy *et al.*, 2007; Sasmal *et al.*, 2007; Roy *et al.*, 2007).

There are already considerable published reports involving the practical use of transition metal complexes as chemical nucleases, anti-candida, antimycobacterial, antimicrobial etc (Dervan, 1986; Dixon *et al.*, 1991; Pyle and Barton, 1990; Sigman, *et al.*, 1993). For example, $[Fe(edta)]^-$ (edta = ethylenediamine tetraacetate) (Dixon *et al.*, 1991) and $[Cu(phen)_2]^{2+}$ (phen = 1, l0-phenanthroline**)** (Sigman, *et al.*, 1993; Sigman *et al.*, 1991), which cleave DNA by an oxidative mechanism, have been used extensively as footprinting reagents to provide insights into protein-DNA interactions. Besides, copper complexes of L-histidine and 1,10-phenanthroline are also reported to exhibit potent antitumor and artificial nuclease activity (Zhang *et al.*, 2004; Ren *et al.*, 2000).

Furthermore, cancer chemotherapy has also involved those transition metal complexes with amino acids as subsidiary ligand (Williams, 1972). L-

amino acids have been widely used in the production of agrochemicals, racemic drugs, fragrances, pharmaceuticals, foods and food addictives (Zhang *et al.*, 2007). Study of interaction of chiral metal complexes with chiral DNA mostly involves the Λ- and Δ-isomers or R- and S-isomers. Those involving D- and L-isomers are less studied. The arrangement, nature of central ions, and the ligand also played important role in the chirality of the metal complexes (Mudasir *et al.*, 2008; Pellegrini Aldrich-Wright, 2003; Kane-Maguire and Wheeler, 2003; Yang and Xiong, 2001). The focus of current study is on optically active metal complexes. The optical activity is a result of the use of chiral amino acids. The studies of Uma *et al.* (2005) showed that copper(II) complexes are attractive in the investigation of artificial nucleases, because these copper(II) complexes can cleave DNA in several ways *viz.* oxidative, photolytic, hydrolytic, and electrolytic cleavage.

All copper(II) complexes (abbreviated as $[Cu(phen)(aa)(H_2O)]X$, X= Cl^-, NO_3^-) used in this study, namely $[Cu(phen)(D\text{-}ala)(H_2O)]Cl$, $[Cu(phen)(L\text{-}ala)(H_2O)]Cl$, $[Cu(phen)(D\text{-}threo)(H_2O)]Cl$, $[Cu(phen)(L\text{-}threo)(H_2O)]Cl$, $[Cu(phen)(D\text{-}threo)(H_2O)]NO_3$, and $[Cu(phen)(L\text{-}threo)(H_2O)]NO_3$ are provided by Assoc. Prof. Dr. Ng Chew Hee from Universiti Tunku Abdul Rahman. The following are the main objectives of this research study:

- To investigate the nucleolytic properties and DNA binding abilities of six different ternary copper(II) complexes with 1,10-phenanthroline and chiral amino acid.
- To study the factors that affects the nucleolytic properties of these complexes on plasmid DNA (pBR322). Agarose gel electrophoresis technique has been used in this section, and those nucleolytic factors investigated are incubation time, complex concentration, type of buffer, pH and presence of exogenous agents (hydrogen peroxide and L-ascorbic acid).
- To compare and differentiate the DNA-binding affinity of the enantiomeric pairs (L-isomer and D-isomer) of the copper complexes with the DNA.
- To study the binding affinity and selectivity towards various types of B-form DNA (i.e., Calf-Thymus (CT) DNA, and oligonucleotides) and G-quadruplex DNA.
- To study the effect of changing the subsidiary ligand on nucleolytic efficiency and DNA binding ability of the complexes.
- To study the human DNA topoisomerase I (topo I) inhibitory effect of above mentioned copper(II) complexes.

CHAPTER 2

LITERATURE REVIEW

2.1 DNA Cleavage Studies

DNA cleavage activity can be defined as reaction that sever one (or more than one) of the covalent sugar-phosphate linkages between nucleotides of the DNA sugar phosphate backbone. In general, DNA cleavage activity can be induced by several agents and catalyzed enzymatically (hydrolytic cleavage), chemically or by radiation. Furthermore, cleavage may be exonucleolytic, i.e., removing the end nucleotide, or endonucleolytic, i.e., splitting the strand in two.

2.1.1 Reactive Oxygen Species (ROS) and cell damage

Eukaryotic cells have to constantly cope with highly reactive oxygen-derived free radicals either produced by the body or from the environment. Their defense against these free radicals is achieved by antioxidant molecules and also by antioxidant enzymes (Genestra, 2007). Generally, free radicals are classified as highly reactive components with unpaired electron and usually present in low concentrations. They react effectively with ubiquitous naturally occurring trapping agents (Chevion, 1988).

In living system, DNA is one of the target sites of Reactive Oxygen Species (ROS) to attack. One very harmful ROS is the hydroxyl radical (·OH). There are already many reports on DNA damage by free radicals produced from the Fenton reaction system or a modified Fenton reaction system involving redox active metal ions (Kawanishi and Yamamoto, 1991; Hirakawa *et al.*, 2003; Kremer, 2003; Yurkova *et al.*, 1999; Lloyd *et al.*, 1998).

$$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + \bullet OH + OH^-$$

$$Fe^{3+} + H_2O_2 \text{ ----> } Fe^{2+} + \bullet OOH + H^+$$

Figure 2.1: The Fenton Reaction system
(Brömme *et. al.*, 2002)

In late 1970s, the transition metals, particularly iron and copper, have been suggested to catalyze the transformation of a superoxide radical anion (O_2^-) to the highly reactive hydroxyl radical (·OH), *via* the Haber-Weiss Reactions (Chevion, 1988).

$$H_2O_2 + OH^- \rightarrow H_2O + O_2^- + H^+$$

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + \bullet OH$$

Figure 2.2: The Haber-Weiss reaction
(Koppenol, 2001)

Oxygen derived ROS species such as superoxide radical, hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radical are well known to be have certain level of cytotoxicity and have been implicated in the etiology of a wide

array of human diseases, including several types of cancer. Various carcinogens may also partly exert their effect by generating ROS during their metabolism (Waris and Ahsan, 2006). DNA lesions resulting from exposure to ROS include modified bases, abasic bases, single and double bond breaks, and DNA-protein crosslinks. Such damage is of potential pathobiological significance, initiation and progression of multistage carcinogenesis, and possibly, initiation of various cancers and ageing. Furthermore, ROS also influences central cellular processes such as proliferation, apoptosis and senescence. They are also implicated in the development of cancer, because many ROS-induced base modifications are pro-mutagenic (Cejudo *et al.*, 2005).

In addition, many studies have indicated a role for ROS in the induction or inhibition of cell proliferation and in both activation and inhibition of apoptosis (Genestra, 2007). At higher concentrations, ROS can also participate in the induction of necrosis. However, although toxicologists have traditionally associated cell death with necrosis, emerging evidence suggests that different types of environmental contaminants exert their toxicity, at least in part, by triggering apoptosis. The mechanism responsible for eliciting the pro-apoptotic effect of a given chemical is often unknown, although in many instances mitochondria appears to be the key participants (Robertson and Orrenius, 2000).

To protect molecules against toxic free radical and other ROS, cells have developed antioxidant defenses by endogenous enzymatic and/or non-enzymatic

components that prevent radical formation, remove radicals before damage can occur, repair oxidative damage, eliminate damage molecules, and prevent mutations (Gordon, 1996).

2.1.2 Transition metal ions and carcinogenesis

Generally, metal ions can be found in various biological systems and they play an important role in our biological systems. Without the catalytic presence of these metal ions in trace or ultra trace amounts, many biochemical reactions would not take place. As an example, metal ion copper is an essential trace element for the human diet. It is required for enzymes and exists in human and animal tissues in biological systems in both +1 and +2 valence states. In addition, the metal ion centers create the binding catalytic sites for biological function or toxicity. Each of the metal ions imposes a specific interaction property to the biological molecule (Theophanides and Anastassopoulou, 2002).

Furthermore, DNA has been reported to contain substantial amounts of copper. Complexation with copper(I) or copper(II) leads to DNA stabilization and destabilization, respectively. Although copper ions have been identified to be involved in DNA stabilization, however, they can change to become toxic to cells when their concentrations surpass certain optimal or natural levels. Catalytically active copper, because of its mobilization and redox activity, is believed to play a central role in the formation of reactive oxygen species (ROS), such as O_2^- and ·OH radicals, that bind very fast to DNA, and produces several types of

damages, such as breaking the DNA strands or modifying the bases and/or deoxyribose, which can lead to carcinogenesis (Theophanides and Anastassopoulou, 2002).

Tumor growth and metastasis are both fully dependent upon angiogenesis, the neovascularization process that requires certain growth factors, proteases, and the trace element copper. Copper, but not other transition metals, is an important co-factor essential for the tumor angiogenesis processes (Brem, 1999; Brewer, 2001; Theophanides and Anastassopoulou, 2002). High levels of copper have been found in many types of human cancers (Chan *et. al.*, 1993; Scanni *et al.*, 1977; Turecky *et al.*, 1984; Zowczak *et al.*, 2001). Copper is important in stimulation of proliferation and also the migration of human endothelial cells (McAuslan and Reilly, 1980; Hu, 1998). A specific amount of local copper appears to be required for angiogenesis to occur (Daniel *et al.*, 2003).

Since metal ions play many important roles in biological system, it is essential to maintain adequate amount of metal ions in the system for the organism to function healthily. Some metals are essential trace elements; however adverse effects are described at higher concentrations. Metals can induce genotoxic effects by affecting the structure of the genetic material (Migliore *et al.*, 1997). World Health Organization (WHO) has classified beryllium, chromium, cadmium, arsenic, and nickel, as carcinogenic elements which will bring certain levels of carcinogenesis effects. The list of definite metallic carcinogens is likely

to be extended in the foreseeable future onto compounds of cobalt, lead, and platinum, and perhaps copper and also iron (Hartwig *et al.*, 1999).

Although nickel, arsenic, cobalt, and cadmium are classified as carcinogenic agents by WHO, however, their mutagenic potentials are rather weak. They exert a so called co-mutagenic effect, which can be explained as disturbances to different DNA repair systems. These elements can get in the way with base and nucleotide excision repair, albeit they affect different step of the respective repair system and act by dissimilar, not yet absolutely understood mechanism (Hartwig and Schwerdtle, 2002).

2.1.3 Design of transition metal-based anticancer drug

The regulation of gene expression is based on the recognition of nucleic acids by repressors, activator, and enhancer proteins. This process is unique and sequence selective because it is usually targeted on a unique or specific sequence. The selective control of such processes has been a long-standing goal, and small molecules that selectively bind DNA and activate (block a repressor) or inhibit (block an activator) gene expression hold significant promise as therapeutics (Boger *et al.*, 2001). Potentially, these molecules could be used as diagnostic probes of nucleic acid structure and function, as well as therapeutic agents for a variety of diseases (Keene *et al.*, 2009).

Drug, broadly speaking, is any chemical substance that, when absorbed into the body of a living organism, alters normal body functions. It is a chemical substance that is used in the treatment, cure, prevention, or diagnosis of disease or used to otherwise enhance physical or mental well-being. Drugs may be prescribed for a limited duration, or on a regular basis for chronic disorders. It is usually distinguished from endogenous biochemical by being introduced from outside the organism and speedily distributed throughout the whole body upon administration, and the interaction of both healthy and cancerous cells and tissues gives rise to the dose-limiting nephro- and hepa-toxicities, as well as to drug resistance (Zutphen and Reedijk, 2005; Giaccone, 2000).

Drug design is an iterative process which begins when a chemist identifies a compound that displays an interesting biological profile and ends when both the activity profile and the chemical synthesis of the new chemical entity are optimized. Traditional approaches to drug discovery rely on a step-wise synthesis and screening program for large numbers of compounds to optimize activity profiles. Over the past ten to twenty years, scientists have used computer models of new chemical entities to help define activity profiles, geometries and reactivities (Richon, 1994).

The interaction and chemical reaction of metal complexes with DNA has long been the subject of intense investigation in relation to the development of new reagents for biotechnology and medicine. Studies of small molecules, which

react at specific sites along a DNA strand as reactive models for protein–nucleic acid interactions, provide routes toward rational drug design as well as means for development of the sensitive chemical probes for DNA. A number of metal chelates have been used as probes of DNA structure in solution, as agents for mediation of strand scission of duplex DNA and as chemotherapeutic agents. However, improvement of anticancer drugs based on intercalating activity is not only focused on DNA-ligand interaction, but also on tissue distribution and toxic side effects on the heart (cardiac toxicity) due to redox reduction of the aromatic rings and subsequent free radical formation. Free radical species are thought to induce destructive cellular events such as enzyme inactivation, DNA strand cleavage and membrane lipid peroxidation (Raja *et al.,* 2005).

2.1.4 The nucleolytic reaction of copper(II) complexes with DNA

Redox active copper complexes are efficient artificial nucleases. It is critically important in a wide variety of catalytic, synthetic, and biochemical processes. Various studies stated that nuclearity is an important factor in the oxidative DNA cleavage, and the synergy between two metal ions contributes to its high nucleolytic efficiency (Karlin *et al.,* 2002).

It is well known that the copper(II) ion cleaves DNA. However, in the studies of Tonde *et al.,* 2005, it is stated that in most of the cases, the cleavage reaction must be initiated by exogenous agents, particularly oxidizing agents, such as hydrogen peroxide (H_2O_2), mercaptopropionic acid, dithiothreitol and also

reducing agent of so called reductant, such as ascorbic acid and so on. In order to induce a more sequence-specific reaction, one should bind stable cupric complexes instead of copper(II) ions to the DNA so that the ligand substituent in the complex recognize a specific base sequence in the DNA (Harada *et. al.*, 1996). The recognition of the base sequence takes place with various interactions such as coordination bonds between metal ions and DNA, hydrogen bonds, electrostatic interactions, and Van der Waals interaction between the ligands and DNA. A subtle balance of these interactions determines the stereo-specific binding models that are classified as intercalative binding, minor or major groove binding, and electrostatic outside binding (Harada *et. al.*, 1996).

In the studies of Lin and Wu (2004), hydrogen peroxide (H_2O_2) is a common oxidizing agent and is often converted to hydroxyl radical (•OH) along with the reduction-oxidation (redox) reaction of transition-metal ions, that is, the Fenton or Fenton-like reaction. It has been reported that the activation of hydrogen peroxide in the presence of transition metal ions is effective only under acidic conditions. At higher pH, a major limitation is precipitation of the catalytically active metal ions. Nevertheless, these metal ions are relatively active when they are chelated as complexes in basic solutions.

Although several reaction mechanisms of metal ion species with hydrogen peroxide have been proposed, a paradigmatic mechanism has not been established. A mechanism, based on Haber–Weiss reaction or Fenton chemistry, assumed that

metal ions were utilized through one-electron redox reactions that convert peroxide into reactive radical species. To understand the activation mechanism of hydrogen peroxide followed by degradation of target substrates, a number of assays of radical species have been developed to distinguish radical chain processes from the others (Lin and Wu, 2004).

Besides that, it is well-known that the ascorbic acid is a powerful antioxidant acting directly *via* scavenging of the ROS, or indirectly through regeneration of other antioxidant systems (Liu *et al.*, 2002). However, under certain conditions, such as the presence of transition metal ions, ascorbic acid would act as a pro-oxidant, which can damage biological molecules, especially nucleic acids. In the ascorbic acid-transition metal ion system, the transition metal ions such as Cu^{2+} and Fe^{3+} should be at a comparatively high concentration. Furthermore, ascorbic acid-copper ion system could work well in the plasmid DNA damage experiments, even at a low Cu^{2+} concentration. Surprisingly, research showed that high concentration of nickel ions could prevent plasmid DNA from being damaged by the ascorbic acid-copper ion system, which was reported for the first time (Liu *et. al.*, 2002).

Although most of the studies shown that the cleavage reaction must be initiated by exogenous agents (i.e. oxidizing or reducing agents), there are some reports stating that self-activating systems that require no further activation to bring about DNA cleavage are desirable under certain conditions. In the studies of

Lamour *et al.* (1999), hydroxysalen-Cu(II) (hydroxysalen = bis(hydroxysalicylidene)ethylenediamine) complexes spontaneously form the oxidant species copper(III) and cleave DNA without an activating agent. Another example is a marine natural product Tambjamine E which induces DNA cleavage in the presence of copper(II) and molecular oxygen without addition of any external reducing agent. Recently, Sissi *et al.* (2005) have reported that the copper(II) complex of the ligand all-*cis*-2,4,6-1,3,5-trihydroxy-cyclohexane is extremely efficient in promoting the cleavage of plasmid DNA under hydrolytic conditions, i.e. in the absence of oxygen and added reducing agents.

Copper(II) complexes are versatile molecules for bringing about DNA cleavage. This is due to the fact that copper(II) complexes not only bring about oxidative cleavage of DNA but also hydrolytic, photolytic and electrolytic cleavage of DNA. Many of the copper(II) complexes explored so far for their nuclease activity, are based on the bidentate 1,10-phenanthroline and its derivatives. These complexes have been utilized as foot printing agents of both proteins and DNA, probes of the dimensions of the minor groove of duplex structure and identifiers of transcription start sites Sigman *et al.* (1979).

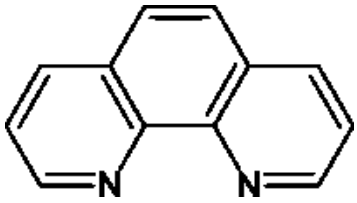


Figure 2.3: Structure of 1,10-phenanthroline

(Sigman *et al.*, 1979)

Nevertheless, Uma *et al.* (2007) also stated that copper(II) complexes of 1,10-phenanthroline have been shown to inhibit DNA and RNA polymerase activities and induce strand scission of DNA in the presence of H_2O_2 or thiol. In this case, the phenanthroline has been shown to influence the reactivity of the complexes with DNA. Because of the fact that copper(II) is a substitution labile metal ion, multi-dentate ligands are believed to be better than bidentate ligands in keeping the copper(II) ion chelated in solution. In addition, recent reports have also shown that amino acid or peptide-based copper(II) complexes show efficient DNA cleavage activity by oxidative and hydrolytic pathways (Rao *et al.*, 2007).

The binding mode of the *bis*-phenanthroline complex to DNA is suggested to involve intercalation or minor groove binding. The nuclease activity of $[Cu(phen)_2]^+$ is related to the partial intercalation or binding of one phenanthroline ligand to the minor groove of DNA while the other phenanthroline ligand makes favorable contact within the groove. The formation of Cu(I)OOH or Cu(III)-OH has earlier been proposed for the nucleobase oxidation involving $CuCl_2$ in presence of H_2O_2 or O_2 with a reductant like ascorbate, reduced glutathione or NADH in DNA strand breaking giving the order T > G > C > A. The 1:1 complex formed by $CuCl_2$ with the anticancer drug famotidine has been shown as a better catalyst than $CuCl_2$ for sulfite auto-oxidation leading to DNA damage. Similarly Cu^{2+} ion and H_2O_2 in the presence of L-dopa or a catechol, greatly enhance DNA oxidation (Chakravarty *et al.*, 2002).

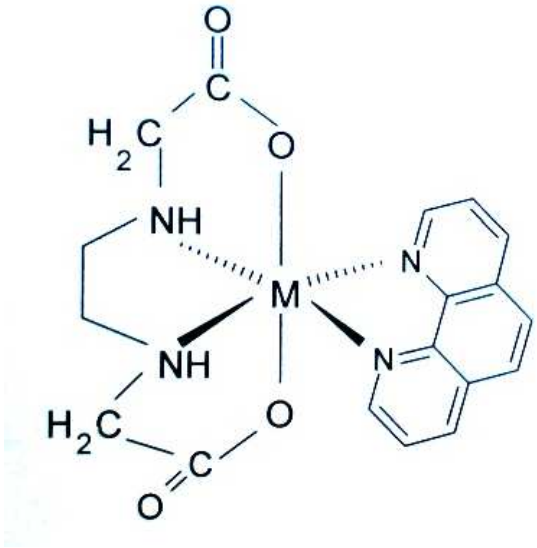


Figure 2.4: General structure of [M(phen)(edda)] complex
(Ng *et. al.*, 2005)

2.1.4.1 Concepts of interaction of metal complexes with DNA

Phosphodiester linkages of DNA are very stable to hydrolysis. The half-life for spontaneous hydrolysis of the phosphodiester has been estimated as about 1011 years at pH 7 and 25 ºC. To design artificial restriction enzymes, it is essential to secure synthetic catalytic centers for effective hydrolysis of the phosphodiester bonds. Hydrolysis of the phosphodiester linkages lead to the conversion of a supercoiled DNA to the corresponding open circular form of DNA. Most of known synthetic catalysts for DNA hydrolysis are metal complexes (Jeung *et. al.*, 2001).

Generally, metal complexes are known to bind to DNA *via* two types of interactions, which are covalent and non-covalent interactions. In covalent

binding, the labile ligand of the complexes is replaced by a nitrogen base of DNA such as guanine which is coordinated through its N7. In fact, cisplatin, a commonly known and important antitumor drug is found to bind to DNA through an intrastrand cross-link between neighboring guanine residues created by covalent binding to two soft purine nitrogen atoms. On the other hand, the non-covalent DNA interactions include intercalative, electrostatic and groove or so called surface binding of cationic metal complexes along the outside of DNA helix, or along the DNA major groove or DNA minor groove. The above mentioned intercalation involves the partial insertion of aromatic heterocyclic rings of ligands between the DNA base pairs (Raja *et. al.,* 2005).

2.1.5 Transition metal complexes as artificial nucleases

The ability to cleave DNA is of paramount interest to the fields of medicine and biotechnology. Owing to their diverse structure and reactivity, transition metal complexes are attractive reagents for the cleavage of nucleic acids (Frey *et al*., 1996). Many of the transition metal complexes can work as chemical nucleases, and many of them can induce effective oxidative cleavage of DNA only in the presence of the UV light, a reducing agent or H_2O_2 as an additive (Tan *et al*., 2005; Zhang *et al*., 2001).

In 1996, Rammo and co-workers found that complexes from Cu, Ni, Cd and Zn with ethylenediamine and propylenediamine derivatives have variable nucleolytic efficiency due to the nature and the redox potential of the metal ions.

Garcýa-Raso and co-workers (2003) had carried out an investigation on three new ternary peptide–copper(II)–1,10-phenanthroline (phen) complexes, [Cu(L-ala–gly)(phen)]•3.5H_2O, [Cu(L-val–gly)(phen)] and [Cu(gly–L-trp)(phen)]•2H_2O. These complexes were structurally characterized and their nuclease properties were also investigated. The results showed that the bulk of the lateral chain in the peptide moiety determines the relative disposition of the phen ligand and these complexes exhibit significant differences in their nuclease activity on DNA which depends on the nature of the peptidic moiety.

Chikira and co-workers (1997) have shown that copper complexes of some simple amino acids could cleave DNA in the presence of hydrogen peroxide, and various side groups of amino acids have a potential to bind specifically to DNA. For example, a guanidine group in arginine is protonated and positively charged in a physiological pH range, consequently, it will also enhance the interaction of the complex with a negatively charged phosphodiester moiety in DNA. In 2002, the research team of Chikira has investigated the orientation of mono(1,10-phenanthroline)copper(II), $[Cu(phen)]^{2+}$, and the ternary complexes with amino acids, $[Cu(phen)X_{aa}]^{m+}$ (where X_{aa} stands for an α-amino acid on DNA fibers). The results suggested that the amino acids in the ternary complexes of glycine, leucine, serine, threonine, cysteine, methionine, and asparagine were partly substituted with some coordinating groups in DNA, whereas the ternary complexes of lysine, arginine, and glutamine remained intact on DNA.

Nevertheless, some transition metal-phenanathroline-amino acid or peptide complexes effectively cleave DNA and these complexes exhibit significant differences in their nuclease activity depending on the nature of the amino acid or peptidic moiety (Barceló-Oliver *et al*., 2007). Ng *et al.* (2006) have reported the nucleolytic property of different metal(II) complexes of *N,N'*-ethylenediaminediacetatic acid and found that *N,N'*-ethylene-bridged amino acids act as tetradentate ligand, in contrast to bidentate simple amino acids. The metal complexes with tetradentate ligand should be more stable in aqueous solutions than the bidentate ligands (Ng *et al*., 2006).

2.1.6 The effect of buffer on nucleolytic property of metal complexes

Generally, buffers are ubiquitous components in most *in vitro* reaction system, due to the reason that they are used to maintain the pH of the solution constant while other components in the reaction mixture are varied. Many of the buffers used to maintain neutral or physiological pH values are based on substituted amines, including the commonly used Tris-acetate and Tris-borate buffers (Sambrook *et al*., 1987) and the zwitterionic "Good" buffers (Good *et al*., 1966). The assumption is usually made that the buffers do not interact with DNA during the reaction. However, this assumption was challenged when some of the researchers found that the electrophoretic mobility of DNA in free solution was found to be about 20 % higher in Tris-Borate-EDTA (TBE) buffer than in Tris-acetate-EDTA (TAE) buffer (Stellwagen *et al*., 1997).

In 1999, Wenner and followers have reported that DNA-buffer interactions also influence the rate of cleavage of plasmid pBR322 by the restriction enzyme *Eco*RV, and this effect has been attributed to differences in the specific and nonspecific binding of the substrate to the DNA in various neutral pH buffers. Wenner *et al.* (1999) proposed that the protonated amines in such buffers act as counter-ions to screen the charge on the phosphate residues, which then affected the binding of the enzyme to the DNA.

In addition, Prenzler *et al*. (1997) have suggested that DNA binding efficiency capable of modifying the ternary structure of pBR322 decreased in the order from $NaClO_4$ to Hepes and Tris, the respective DNA binding efficiencies nicely paralleled the increasing potential of buffer systems components to coordinate transition metal complexes that compete with DNA for binding to metal.

Furthermore, previous studies found that cisplatin dissolved in water to form *cis*-$[Pt(NH_3)_2(H_2O)(Cl)]^+$ which subsequently attacks DNA with the loss of bound water in Hepes buffer and in other media having poor metal binding ligands. This initially formed mono-functional adduct ultimately losses the chloride ligand to form a bifunctional intrastrand crosslink, which changes the degree of supercoiling. Hence, the reduction of fluorescence in Hepes could be caused by the ability of cationic platinum adducts to reduce binding of cationic ethidium near the lesion and to platinum induced structural changes in DNA

which hinder intercalation of ethidium (Binter *et al*., 2006). From this case, it is clearly shown that the composition of medium or buffer significantly influences the outcome of the binding experiment.

Nevertheless, detailed studies with soluble or membrane-bound enzymes and cultured cell lines have shown these buffers to be often superior to inorganic buffers, such as phosphate, borate and bicarbonate buffers, in protecting many systems from denaturation. Thus, the organic buffers have also been used in examination of the formation and effects of free radicals in biological systems (Zhao *et al*., 2006).

2.2 DNA-binding studies

A variety of small molecules, such as drugs, dyes, metals and some other components, can bind to nucleic acids. This binding will affect the normal function of nucleic acids, and different type of binding will result in different type of downstream processes. Since DNA is a target of anticancer treatment as well as other diseases, it is very important to characterize the binding affinity and to understand the mechanism of DNA-drug interactions.

2.2.1 The importance of DNA-binding study

The binding between DNA and metal complexes has attracted interest over the past ten years. DNA intercalating agents disrupt the normal function of cellular DNA and can lead to interference with gene expression, gene transcription, mutagenesis, carcinogenesis, and cell death. Hence, a precise understanding of the DNA-binding properties of metal complexes is very important in medicinal and pharmaceutical fields. Most of the studies are directed toward the design of site and conformation specific reagents to provide routes toward rational drug design as well as a means to develop sensitive chemical probes of DNA (Metcalfe and Thomas, 2003; Barton *et al*., 1984; Becke, 1993; Gorling, 1996; Coggan *et al*., 1999).

DNA has been proposed to have a number of types of sites in which a molecule might bind: (i) between two base pairs (intercalation), (ii) in the minor groove, (iii) in the major groove, and (iv) on the outside of the helix (Nakabayashi

et al., 2004). The interaction can be studied by using various spectrometric techniques, such as NMR, ESR, circular dichroism (CD), fluorescence, resonance Raman, UV-visible and Fourier transform infrared (FTIR) spectroscopy, as well as electrochemical method. Among these spectra, the signals of the absorption and induced CD (ICD) spectra of small molecules bound to nuclei acid provide conveniently a signature for the binding mode to DNA (Jiang *et al*., 2005; Selvakumar *et al.*, 2006). Another example is given by the intercalation of ethidium bromide into DNA which act as a fluorescence probe for DNA and has recently been employed in examination of the torsional rigidity of the double helix (Kumar *et al*., 1993).

2.2.1.1 Fluorescence Intercalator Displacement (FID) Assay

The fluorescence intercalator displacement (FID) assay has proven to be inexpensive, rapid and accurate for DNA-binding studies. This method is commonly used to study both organic molecules and metal complexes (Boger and Tse, 2001).

Ethidium bromide (EB) is a probe for DNA structure detection. Generally, competitive ethidium bromide binding study was undertaken to understand the mode of interaction between DNA and the metal complexes. The molecular fluorophore EB emits intense fluorescence in the presence of calf-thymus (CT) DNA due to its strong intercalation between the adjacent DNA base pairs. With the addition of a second molecule, which may bind to DNA more strongly than

EB, this second molecule would quench the DNA-induced EB emission (Liu *et al.*, 1998). If a complex could replace EB from DNA-bound EB, the fluorescence of the solution would be greatly quenched due to the fact that the free EB molecules were readily quenched by the surrounding water molecules (Lacowicz and Webber, 1973). Moreover, two mechanisms have been proposed to account for the quenching of EB emission, viz the replacement of the molecular fluorophores and electron transfer (Raja *et al.*, 2005).

Addition of a DNA binding compound results in a decrease in fluorescence due to the displacement of the bound intercalator. The % fluorescence decrease is directly related to the extent of DNA binding, providing relative binding affinities and a rank order binding to all possible five or four base pair (bp) sequences (Boger *et al.*, 2001). In 2001, Song and Yang reported that the fluorescence intensity is highly increased when [Co(phen)$_2$phendione]Cl and [Co(phen)$_2$tpphz]Cl (tpphz = planar ligand tetrapyrido[3,2-a:2’,3’-c:3’’,2’’-h:2’’’,3’’’-j]phenazine) intercalated into the base pairs of DNA. This can be proven when the ratio [DNA]/ [EB] = 0.5 and 2.2 was used. After the addition of these two complexes, the fluorescence intensity was increased 1.7 and 8.5 times of that of EB alone, respectively.

As another example of the FID assay, the synchronous fluorescence spectroscopy was applied to investigate the competitive interaction of DNA with the bis(1,10-phenanthroline)copper(II) complex cation ([Cu(phen)$_2$]$^{2+}$) and a

fluorescence probe, neutral red dye (NR). The results showed that the excitation fluorescence spectrum of the NR-DNA intercalated complex decreased in intensity on the addition of $[Cu(phen)_2]^{2+}$, indicating that the intercalated species, $[Cu(phen)_2]^{2+}$-DNA, quenched fluorescence (Ni *et al*., 2006).

2.2.1.2 DNA melting profile

DNA denaturation, or commonly known as DNA melting, is the process by which double-stranded deoxyribonucleic acid (DNA) unwinds and separates into single-stranded strands through the breaking of hydrogen bonding between the bases. Both terms are used to refer to the process as it occurs when a mixture is heated, although "denaturation" can also refer to the separation of DNA strands induced by chemicals like urea. For multiple copies of DNA molecules, the melting temperature, T_m, is defined as the temperature at which half of the DNA strands are in the double-helical state and half are in the "random-coil" states. T_m also can be defined as physical property of nucleic acids that gives information about the stability of duplexes in a specified environment. T_m values are very useful in a variety of fields ranging from practical assay design in molecular biology to theoretical biophysics and it is fully depending on both the length of the molecule, and the specific nucleotide sequence composition of that molecule (Owczarzy, 2005).

Some researchers found that the binding of organic dyes or metallo-intercalators generally results in a stabilization of the DNA duplex with

corresponding increase in T_m. In the presence of intercalators, the T_m rises sharply until all the intercalating sites are saturated, whereas the T_m increases less steeply if the stabilization is caused by electrostatic binding (Neyhart *et al*., 1993). Besides, thermal behavior of DNA in the presence of metal complexes can give insight into their conformational changes when the temperature is raised and information about the interaction strength of the complexes with DNA, and a minor increase in the melting temperature of ~5°C suggesting primarily electrostatic and/or groove binding nature of the complexes (Patra *et al*., 2007).

From the report of Portugal José (1989), it is stated that the derivative denaturation profiles of calf thymus DNA in the presence of copper(II) ions have been directly obtained from high resolution thermal denaturation profiles recorded in an iso-absorbance wavelength of the AT and GC hyperchromic spectra. The analysis of the very sensitive profiles provides further evidence that the T_m of DNA decreases in the presence of stoichiometric ratio of copper(II) ions to nucleotide. Also, evidence is given of peculiar behavior at higher temperatures where a new melting transition is observed. This phenomenon could be in line with the presence of bridging of DNA single strands by copper ions which are disrupted when the temperature is raised.

2.2.2 Binding modes

The transition metal complex can interact non-covalently with DNA by intercalation, groove-face binding or external electrostatic binding. Different types of binding will bring different effects and downstream processes. For example, it may induce intercalation between stacked base pairs, distorting the DNA backbone conformation and therefore, affect the DNA-protein interaction (Liu *et al.*, 2002).

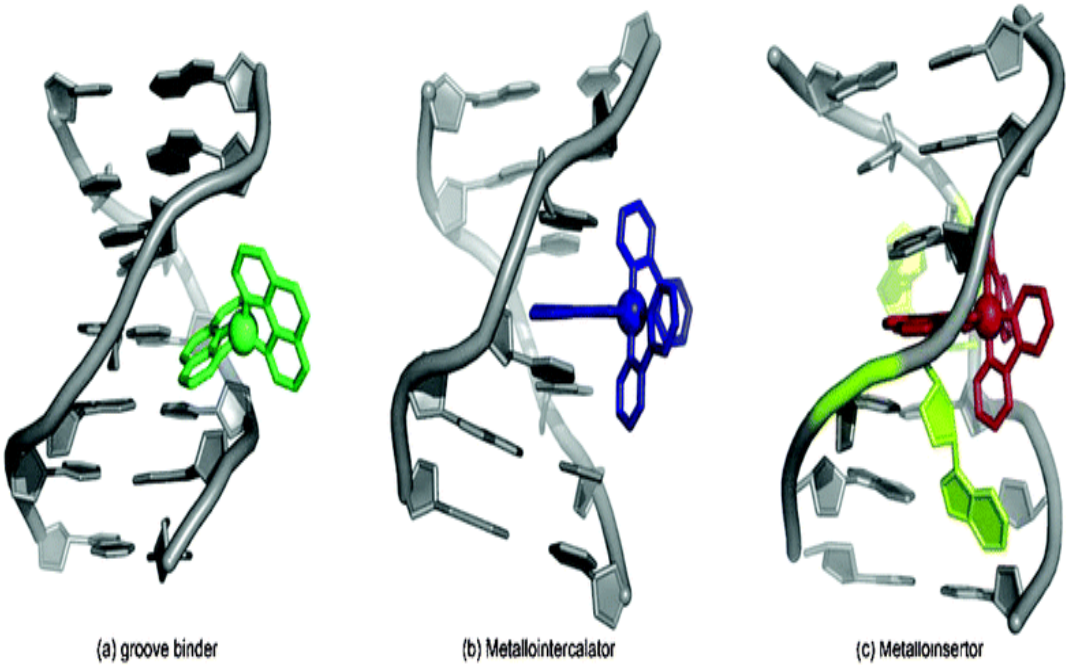


Figure 2.5: The three binding modes of metal complexes with DNA: (a) groove binding, (b) intercalation, and (c) insertion (Raja et al., 2005)

2.2.2.1 Site specific binding mechanism

As stated in the report of Chevion (1988), metal ions (eg. Cu^{2+}, Fe^{2+}) that are bound to biological molecules can undergo cyclic reduction and re-oxidation. The reducing agents such as superoxide radical anion (O_2^-), ascorbate, isouramil, glutathione, and others could reduce the metallic complex yielding cupro or ferro states. Subsequently, these reduced states can react with H_2O_2 in the *Fenton* reaction yielding the hydroxyl radical. Site specific metal mediated mechanism explained that the funneling of free radical damage to specific sites, where the metal is bound. Relatively, unreactive reducing agents such as superoxide or ascorbate, whose life span is comparatively long, can migrate a relative long way until they encounter a redox active metal and react with it.

In addition, this site specific mechanism also explained that the transformation of rather benign and unreactive species, such as superoxide or ascorbate to the highly reactive hydroxyl radical, which is known to cause a variety of molecular disruptions. Generally, the hydroxyl radical is characterized by very high kinetic constants for its reaction with a variety of biological molecules or residues. This radical can act by causing breaks in the polymeric backbone of a macromolecule, by abstracting a hydrogen atom or by adding to a double bond (Halliwell and Gutteridge, 1984).

Lastly, site specific mechanism also stated the possible “multi-hit” effect that is observed. Alternatively, repeated cycles of reduction and re-oxidation can

take place at the same fixed locus by rapidly exchanging transition metals. Because of the high reactivity; it is likely that the hydroxyl radical will inflict its damage within a few encounters or near the site of its formation – the metal binding site (Czapski, 1984). This clearly showed that in the site-specific mechanism, there could be either an amplification or a dampening of the effects of a given number of hydroxyl radicals, compared with the effect of statistically and evenly distributed hydroxyls that would cause damage by random hits (Gutteridge and Wilkins, 1983; Gutteridge, 1984; Czapski, 1984).

2.2.2.2 Inner-sphere coordination

Inner sphere coordination is a type of surface binding between molecules and DNA. Inner sphere coordination occurs when ions bind directly to the surface with no intervening water molecules. These types of coordination are restricted to ions that have a high affinity for surface sites and include specifically adsorbed ions that can bind to the surface through covalent bonding (Taube *et al.*, 1953).

Inner-sphere coordination or bonded electron transfer proceeds *via* a covalent linkage between the two redox partners, the oxidant and the reductant. In inner-sphere electron transfer, a ligand bridges the two metal redox centers during the electron transfer event. Inner sphere reactions are inhibited by large ligands, which prevent the formation of the crucial bridged intermediate. Thus, inner-sphere electron transfer is rare in biological systems, where redox sites are often shielded by bulky proteins (Taube *et al.*, 1953).

Several compositional variants of cisplatin, including modification of the amine donors as well as replacement of the labile chloride ligands with carboxylates, are active and currently in clinical trials (Figure 2.6). Advanced ligand designs to cisplatin frameworks, in hopes of enhancing transport properties and targeting, as well as generating antitumor function to cisplatin-resistant cell lines (Komeda *et al.*, 2002; Kalayda *et al.*, 2004), are at the frontier of the field. Furthermore, more recent advances in ligand design include the use of 'bone-seeking' [(bis(phosphonomethyl)aminoκ*N*)-acetato-κ*O*]$^{2-}$ ligands that have a high affinity for the hydroxylapatite bone matrix to combat osteosarcoma and bone metastases (Galanski *et al.*, 2003).

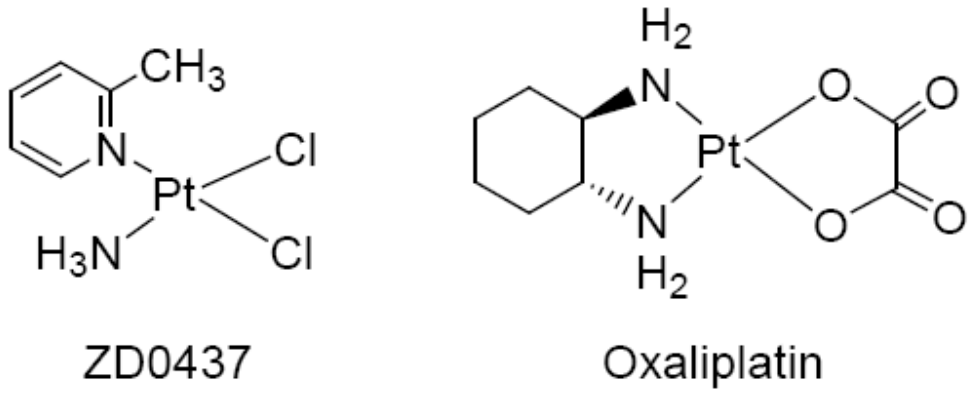


Figure 2.6: Cisplatin antitumor analogues in clinical trials (Boerner and Zaleski, 2005)

2.2.2.3 Intercalative binding

Intercalative binding is one of the ways of molecules to interact with DNA. Intercalation occurs when ligands of an appropriate size and chemical nature fit themselves in between base pairs of DNA.

The combined advantages of inner-sphere DNA binding and optical properties of an intercalative inert chiral framework have recently been grafted into a single molecular construct toward the development of multifunctional supra-molecular complexes (Williams *et al.*, 2003). These types of supra-molecular complexes with variable linker lengths and conformations are proposed to bind to DNA as a two-point intercalative biopolymer chelate. Furthermore, the constructs of these types of complexes will undoubtedly become more prominent as DNA binding approaches evolve toward synthetic metallo-restriction enzymes (Kurosaki *et al.*, 2003).

2.2.2.4 Major and minor groove binding

Intense interest exists in the design and synthesis of small molecules that might selectively bind to the defined sites in DNA or RNA (Chaires, 1998). Targeting particular sequences within the right-handed, B-form DNA is one approach to producing the desired selectivity (Dervan, 1986). Sequence selectivity might exploit the unique, sequence-dependent patterns of hydrogen bond donors and acceptors within the major and minor grooves of DNA. The

design of ligands capable of sequence-specific DNA binding was recently realized by Dervan group with the development of the hairpin polyamides (Trauger *et al.*, 1996; Wemmer and Dervan, 1997; White *et al.*, 1998). The recognition code for the hairpin polyamides was elucidated (White *et al.*, 1998), and the effectiveness of these molecules as selective inhibitors of gene expression *in vivo* was demonstrated (Gottesfeld *et al.*, 1997).

Furthermore, the research work on pyrrolopeptide-based recognition agents of the DNA minor groove was pioneered in Professor Dervan's laboratory (Wemmer *et al.*, 1997; White *et al.*, 1998; Dervan and Burli, 1999). Attachment of the radical cleaving moiety, MPE·Fe (II), to the pyrrolopeptide-based agents led to the design of sequence specific DNA cleaving agents (Dyke *et al.*, 1982; Herzberg and Dervan, 1984; Taylor *et al.*, 1984). In addition, the field of DNA minor groove recognition has grown with many investigators and with many variations of the pyrrolopeptide structure (Cozzi, 2000; Denny, 2000; Fox *et al.*, 1999; Guelev *et al.*, 2000; Hamy *et al.*, 2000; Lida *et al.*, 1999).

The DNA major groove binding approach has also been used for the design of sequence specific recognition agents. Usually DNA strands or synthetic analogues thereof recognize the major groove by Hoogsteen base pairing to afford triple helix structure (Nielsen, 2000; Majumdar *et al.*, 1998; Thurston, 1999; Ren and Chaires, 1999; Crooke, 1999). Because the understanding of DNA recognition at the minor and major groove is important in cancer chemotherapy

and molecular biology, efforts in this area will no doubt continue into the new millennium (Thurston, 1999).

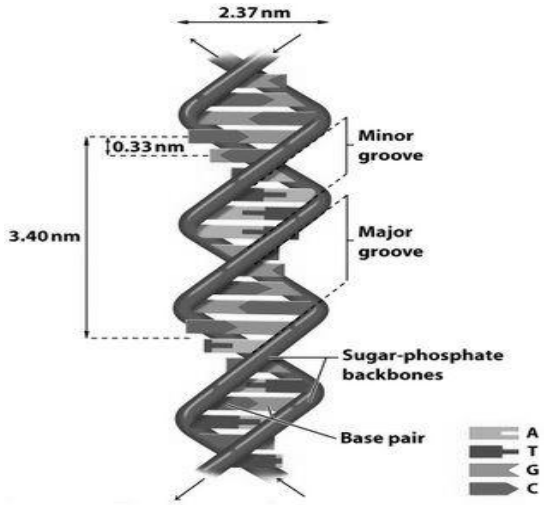


Figure 2.7: The minor and major groove of DNA (Watson and Crick, 1953)

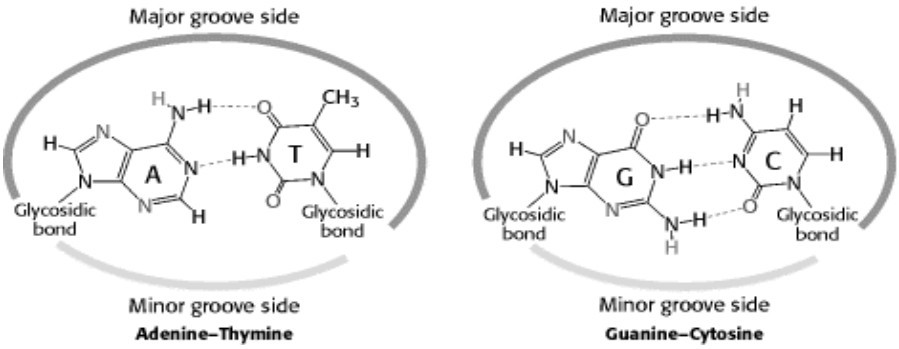


Figure 2.8: Major and Minor Groove Sides. Because the two glycosidic bonds are not diametrically opposite each other, each base pair has a larger side that defines the major groove and a smaller side that defines the minor groove (Bailly and Waring, 1998).

2.2.3 Chirality and Enantiomerism

Chirality can be defined as a property of molecules having a non-super-imposable mirror image. Two mirror images of a chiral molecule are called enantiomers or optical isomers. Pairs of enantiomers are often designated as "right-" and "left-handed." Molecular chirality is of interest because of its application to stereochemistry in inorganic chemistry, organic chemistry, physical chemistry, biochemistry, and supra-molecular chemistry (Bruice, 2004).

Normally, the two enantiomers of a molecule behave similarly to each other; they might possess similar biological and chemical properties in certain way. However, enantiomers behave differently in the presence of other chiral molecules or objects. For example, enantiomers do not migrate identically on chiral chromatographic media, such as quartz or standard media that have been chirally modified. The NMR spectra of enantiomers are affected differently by single-enantiomer chiral additives such as $Eu(fod)_3$ ($Eu(OCC(CH_3)_3CHCOC_3F_7)_3$). In addition, chiral compounds rotate plane polarized light. Each enantiomer will rotate the light in a different sense, clockwise or counterclockwise. Molecules that do this are said to be optically active (Fox and Whitesell, 2004).

Chirality is known to play an important role in biological system. Chirality is known to enhance the pharmacological behavior of metal complexes and asymmetric substituents lead to different biological activity for two enantiomers (Benedetti *et al*., 2002). Different enantiomers of a chiral metal complex are

expected to show different metallo-intercalation capacity with DNA molecules which are essentially chiral in nature (Shi *et al*., 2008). In addition, the design and synthesis of chiral ligands that upon coordination with metal ions can induce high stereo-selectivity for organic transformations constitute an important issue in modern coordination chemistry (Che and Huang, 2003).

The DNA double helix has a chiral structure, and complexes with enantiomeric amines as ligands may therefore lead to different diastereomeric interactions with DNA (Inagaki and Kidani, 1986; Cerasino *et al*., 1997). Moreover, Hegstrom and Kondepudi (1990) also suggested that the selective binding of chiral molecules is regiodirectional and in pharmaceuticals, one chiral form is more dominant than another. As an example, R and S enantiomers of Pt(II) complexes show differences *in vitro* cytotoxic activities in some of cell lines studied with Salmen Sperm DNA, and as a result of the markedly different behaviour of the two enantiomer forms, only R,R enantiomer of [Pt(DACH)(oxalato)] (abbreviated as oxaliplatin) has been approved for clinical use (Misset, 1998).

Besides that, the intercalation of ligand L of the complex L-$[Co(phen)_2dpq]^{3+}$ into DNA base stack has been emphasized on enantio-selectivity, site-specificity and binding mechanism etc (Proudfoot, 2001; Bhattacharya, 2003; Dupureur and Barton, 1994; Collions, 1999; Dupureur and Barton, 1997; Rehman and Barton, 1990). Wu and friends (2005) also suggested

that the L-isomer of cobalt(II) complex will preferentially recognize the right hand double helical structure of the oligodeoxynucleotide. In studying the racemic D,L -$[Co(phen)_2dpq]^{3+}$ - DNA interactions (dpq = bidentate ligand dipyrido [3,2-d:2,3-f]quinoxaline), the selective intercalation of cobalt(II) complex to the DNA base stack with dpq as intercalator were observed. This recognition event show obvious enantio-selectivity, site-specificity and groove-selectivity. L-$[Co(phen)_2dpq]^{3+}$ recognizes pyrimidine–purine/ purine–pyrimidine region, especially CG/GC sequence, while the D-isomer recognizes the pyrimidine–pyrimidine/ purine–purine region, especially TC/AG sequence. In 1993, Satyanarayana and co-workers has investigated the sequence-related enantio-selectivity of $[Ru(phen)_3]^{2+}$ by using competition dialysis . They reported that the Δ-enantiomer interacted preferentially with GC-rich DNA and Λ enantiomer with the AT-rich DNA.

In a different report, it has been concluded that each enantiomer had only one binding mode (Hiort *et al*., 1990). Binding mode was different between enantiomers, with Δ-isomer favoring a location with two chelates in the major groove and Λ-isomer having a single, non-intercalated chelate in the major groove parallel to the bases of DNA. Sun *et al*. (2009) had synthesized both the Δ- and Λ-enantiomers of $[Ru(bpy)_2(nfip)]^{2+}$ (bpy = 2,2'-bipyridine, nfip = 2-(5-nitrofuran-2-yl)-1H-imidazo[4,5-f][1,10]phenanthroline) for the study of DNA-binding properties. A subtle but detectable difference was observed in the interaction of both enantiomers with CT-DNA, where Δ enantiomer binds with DNA more

strongly than Λ enantiomer. Viscosity experiments provided evidence that both Δ-$[Ru(bpy)_2(nfip)]^{2+}$ and Λ-$[Ru(bpy)_2(nfip)]^{2+}$ bound to DNA by intercalation.

Last but not least, the research group of Foley (2001) reported that the ΔΔ–enantiomer interacted differently with the $d(CAATCCGGATTG)_2$ dodecanucleotide compared with the ΛΛ–enantiomer, as shown by the different nuclear magnetic resonance (NMR) spectral changes in the chemical shift of the resonances from the enantiomers upon binding. Addition of the *meso*- (ΔΛ) isomer to $d(CAATCCGGATTG)_2$ resulted in a doubling of the resonances from the metal complex: one set of resonances from the ΔΛ-isomer, while the other set of resonances exhibited shifts consistent with those observed upon addition of the ΔΛ-isomer to $d(CAATCCGGATTG)_2$. This suggests that the monomeric units display enantio-selective binding even when part of the same molecule. Furthermore, the binding geometry of each monomeric unit appears to be dependent upon its absolute configuration and not upon the interaction of the two units as a whole. A similar observation was made by Önfelt *et al.* (1999) for the DNA binding of dinuclear intercalating complex $[\{Ru(phen)_2\}_2\{\mu\text{-}c4(cpdppz)_2\}]^{4+}$ (c4(cpdppz)$_2$ = *N*,*N*'-bis(12-cyano-12,13-dihydro-11*H*-cyclopenta[*b*]dipyrido[3,2-*h*:2',3'-*j*]-phenazine-12-carbonyl)-1,4-diaminobutane).

As can be seen above, D- and L-isomers of metal complexes are less studied for their interaction with DNA or other biomolecules.

2.3 G-quadruplex DNA

Study of interaction of various compounds with DNA now includes G-quadruplex DNA, a non B-form DNA. However, previously it mostly involves only B-form DNA.

G-quadruplexes, also known as G-tetrads or G4-DNA, are four-stranded nucleic acid structures which are rich in guanine. These consist of a square arrangement of guanines (a tetrad), stabilized by Hoogsteen hydrogen bonding. They are further stabilized by the existence of a monovalent cation (especially potassium) in the center of the tetrads. Depending on the direction of the strands or parts of a strand that form the tetrads, structures may be described as parallel or anti-parallel.

2.3.1 Telomere, telomerase and G-quadruplex DNA

Telomere is a region of repetitive DNA sequences that capped the ends of all eukaryotic chromosomes, and they play crucial roles in maintaining genomic stability by providing both end-protection and a mechanism for generating chromosomal ends (LeBel and Wellinger, 2005). Telomeres protect the chromosome ends from degradation, recombination and DNA repair activities (Blackburn, 1991; Greider, 1996). In addition, telomere length is progressively reduced with cell divisions, due to the “end-replication problem” and the putative exonuclease activity in the CA-rich strand. Telomere shortening in aging cells induces replicative senescence (Reddel, 2000; der-Sarkissian *et al*., 2004). When

telomere lengths reach a critical size, chromosomes become unstable and undergo end-to-end fusions, DNA fragmentation, and mutations (Blackburn, 2000, 2001).

Generally, human telomeric DNA composed of $(TTAGGG/CCCTAA)_n$ repeats and it may fold into a four-stranded quadruplex structure. This structure is an attractive potential structure-specific target in rapidly dividing cells, such as eukaryotic parasites and cancer (Cech, 1988; Phan and Mergny, 2002; Makarov *et al*., 1997; Ren *et al*., 2002; Wang and Patel, 1993, Parkinson *et al*., 2002). The key structural feature of a quadruplex is a series of stacked guanine tetrads held together in a coplanar cyclic array by Hoogsteen and Watson-Crick hydrogen bonds. Each individual strand has the propensity to form four-stranded structures (Figure 2.9). The G-rich strand adopts a G-quadruplex conformation stabilized by G-quartets while the C-rich strand may fold into an I-motif based on intercalated $C \cdot C^+$ base pairs (Phan and Mergny, 2002; Makarov *et al*., 1997; Ren *et al*., 2002; Wang and Patel, 1993, Parkinson *et al*., 2002).

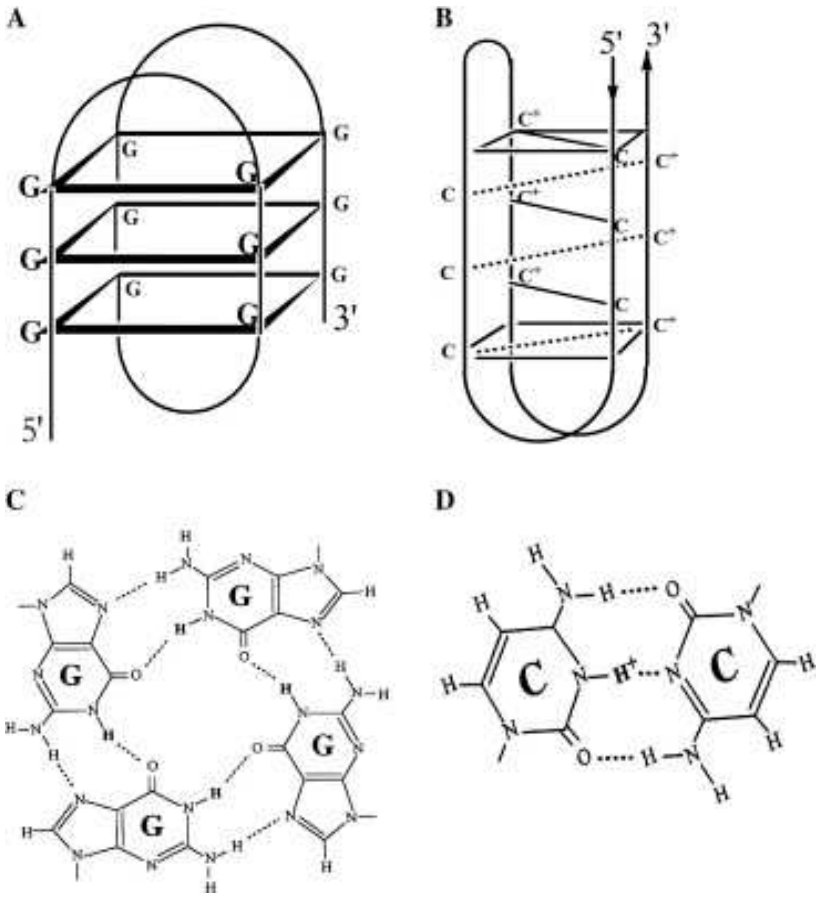


Figure 2.9: Folded structure of the G-rich strand (A) and C-rich strand (B) of human telomeric DNA; C: the G-quartet; D: the C.C+ hemiprotonated base pair of the "Building blocks" for quadruplex formation (Xu *et al.*, 2006)

The quadruplex is also stabilized through π–π stacking interactions of the stacked tetrads as well as by coordination with cations located between or within the tetrads. Guanine-rich sequences, which are capable of forming quadruplex structures, are present in biologically significant regions of the genome including immunoglobulin switch regions (Sen and Gilbert, 1988), the transcriptional regulatory regions of a number of genes such as the insulin gene (Catasti *et al.*,

1996), and also the promoter regions of certain oncogenes, such as c-MYC (Siddiqui-Jain *et al*., 2002; Simonsson, 1998).

Most telomeric DNA consists of simple repetitive sequences with G-rich termini (Phan and Mergny, 2002; Makarov *et al*., 1997). The telomeric DNA has a unique mode of replication based on a special reverse transcriptase-like enzyme called telomerase (Neidle and Parkinson, 2002). Telomerase is the most important enzyme involved in telomere maintenance in tumor cells identified so far (Masutomi *et al*., 2003; Wong and Collins, 2003). It is a large RNA-dependent DNA polymerase that uses its own associated RNA template to catalyse the addition of telomeric DNA repeats to the 3'-end of the single-stranded DNA telomere (Feng *et al*., 1995; Nakamura *et al*., 1997). Moreover, cancer cells often expressed high levels of telomerase while the somatic cells expressed low level. This evidence suggested that telomerase inhibitors may be a powerful new approach to cancer chemotherapy (Kim *et al*., 1994; Greider, 1996). In addition, a small proportion of tumor cells have an alternative telomere maintenance pathway (alternative lengthening of telomeres, ALT), which appears to be independent of telomere and involves recombination events (Bryan *et al*., 1997; Dunham *et al*., 2000; Read *et al*., 2001; Schwarze *et al*., 2001).

Because of the critically essential roles of telomere DNAs in both cancer and parasitic cells, the telomere is a particularly attractive target for drug design (Incles, 2003; Kelland, 2005; Mergny *et al*., 2002; Olaussen *et al*., 2006; Rezler *et*

al., 2002; Rezler *et al*., 2003; Saretzki, 2003; Ya, 2005). The telomeric sequence and structure varies depending on the organism. In humans and other vertebrates and the eukaryotic parasites, telomeres consist of tandem T_2AG_3 repeats that can adopt a G-quadruplex conformation *in vitro* under physiological conditions (Parkinson, 2002; Wang and Patel, 1993). The discovery of proteins such as transcription factors, nucleases and helicases that can bind to and even promote the formation of telomeric quadruplexes suggests that these structures may exist *in vivo* under certain conditions (Fang and Cech, 1993; Giraldo *et al*., 1994). A very exciting recent finding is that a radio-labeled G-quadruplex binding ligand accumulated in nuclei of cultured cells and preferentially bound to the terminal regions of the chromosomes, indicating that G-quadruplexes do exist *in vivo* and are accessible to drugs (Granotier *et al*., 2005).

The formation of higher ordered structures such as G-quadruplexes prevents hybridization of the telomerase RNA template onto the primer and inhibits telomerase activity through an indirect topological mechanism (Zahler and Williamson, 1991). Regardless of the existence of native human quadruplexes, they have recently received attention in the context of telomerase inhibition as a potential anticancer therapy (Rezier *et al*., 2003; Neidle and Parkinson, 2002). This may due to the reasons that G-quadruplex DNA plays a functional role in transcription of the proto-oncogene *c-myc,* and the presence of potential quadruplex forming sequences in the promoter sites of *PDGF-A, c-abl, c-feslfps, c-myb, c-src, c-ets, c-yes,* and *c-vav* which are vital in gene regulation.

Furthermore, strong indication of the *in vivo* presence of quadruplex motif and its functional role comes from the transcription inhibition of *c-myc* in HeLa cells by a quadruplex-specific small molecule. Success in arresting expression of *c-myc* in cancerous cells makes G-quadruplex structures an interesting candidate for design of potential drugs (Arcinas *et al*., 1994; Arthanari *et al*., 1994).

Nevertheless, compounds that have been shown to bind to quadruplex DNA have traditionally been planar, aromatic compounds that bind *via* external end-stacking to the G-quartet on either one end or both ends of the quadruplex (Incles, 2003; Kelland, 2005; Mergny *et al*., 2002; Olaussen *et al*., 2006; Rezler *et al*., 2002; Rezler *et al*., 2003; Saretzki, 2003; Ya, 2005). These compounds, which include anthraquinones, cationic porphyrins, acridines, macrocyclic compounds and analogs, have planar aromatic surface areas that mimic the large planar surface of the G-tetrads in quadruplex DNA (Fedoroff *et al*., 1998; Han *et al*., 1999; Read *et al*., 2001; Teulade-Fichou, 2003). Since essentially all known quadruplex DNA binders are based on, or derived from duplex intercalator, many exhibits little selectivity for quadruplex over duplex structures and this can result in nonspecific cytotoxicity. Increasing the selectivity of telomerase inhibitors for their quadruplex targets is an important focus of research (White *et al*., 2007).

2.3.2 G-quadruplex DNA binding study

Folding of the telomeric G-rich single strand $(GGGTTA)_n$ into G-quadruplex DNA has been found to inhibit telomerase activity (Zahler *et al*., 1991). It was deduced from this observation that a molecule that favours G-quadruplex formation locks the telomeric substrate into an inactive confirmation that is no longer recognized nor extended by the telomerase (Sun *et al*., 1997). Stabilization of G-quadruplexes can then be considered a strategy to achieve antitumor activity (Mergny and Hélène, 1998). G-quadruplexes ligands require a structural selectivity (i. e., preferential binding to G-quadruplexes over duplexes and single strands). The quadruplex itself which is very different from classical double-stranded B-DNA, provides a good structural basis for selective recognition, and several classes of small molecules that selectively bind to G-quadruplex DNA and inhibit telomerase activity (Rosu *et al*., 2003).

In general, small molecules that stabilize G-quadruplex structures have been found to be effective telomerase inhibitors, and the use of drugs to target G-quadruplexes is emerging as a promising way to interfere with telomere replication in the tumor cells and to act as anticancer agents (Alberti *et al*., 2003; Riou, 2004). In fact, a number of small molecules have been reported in recent years that stabilize G-quadruplex structures and inhibit telomerase activity. Some of these molecules have been shown to induce telomere shortening and instability, triggering apoptosis in various tumor cell lines (Galezowska *et al*., 2007).

In 2007, Galezowska and co-workers reported the interactions of G-quadruplex DNA with oxidation products of papaverine, 6a,12a-diazadibenzo-[a.g]fluorenylium derivative and 2,3,9,10-tetramethoxy-12oxo-12H-indolo[2,1-a]isoquinolium cation. Both ligands were capable of interacting with G-quadruplex DNA with binding stoichiometry indicating that two ligand molecules bind to G-quadruplex, which agrees with the binding model of end-stacking on terminal G-tetrads. Furthermore, circular dichroism spectra revealed that preferences of quadruplex-forming oligonucleotide to adopt a particular topological structure may also be affected by the external ligand that binds to quadruplex (Galezowska *et al*., 2007). Besides that, a group of researchers confirmed that two marine compounds, ascididemin and meridine are not just able to interact with DNA but also recognize triplex and quadruplex structures. Interestingly, these molecules exhibit a significant preference for quadruplexes over duplexes or single-strands by dialysis competition assay and mass spectrometry experiments (Guittat *et al*., 2005).

The stabilization of different G-quadruplex intra- and intermolecular structures by a number of perylene derivatives characterized by side chains ending with linear or cyclic amines was investigated. Derivatives carrying a cyclic amine in the side chains, show reduced binding to the G-quadruplex form, while linear amine congeners, exhibit enhanced affinity. The latter efficiently induce pairing of multiple DNA chains, while the former are not able to overcome the original folding of the nuclei acid sequence which is preserved in the complex. In

fact, linear alkylamino terminals in the perylene side chains are capable of strong and selective G-quadruplex recognition, but only cyclic amine end groups favor duplex-quadruplex transitions that are likely crucial to produce biological and pharmacological effects in living systems (Pivetta *et al*., 2008).

Recently, a series of new 9-O-substituted berberine derivatives (Figure 2.10) as telomeric quadruplex binding ligands were synthesized and evaluated. The results from biophysical and biochemical assay indicated that introducing positive charged aza-aromatic terminal group into the side chain of 9-position of berberine significantly improved the binding ability with G-quadruplex, and exhibited the inhibitory effect on the hybridization and on telomerase activity. In addition, these derivatives showed excellent selectivity for telomeric G-quadruplex DNA over duplex (Ma *et al*., 2009).

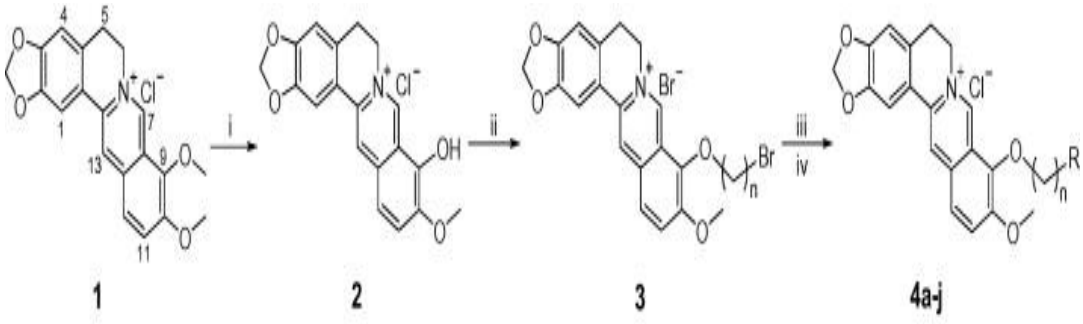


Figure 2.10: 9-O-substituted berberine derivatives
(Ma *et al*., 2009)

Other than organic compounds, several groups have utilized transition metal complexes to develop quadruplex-binding agents. Basically, metal complexes ideally need to fulfill some basic criteria of a good quadruplex binding compound, viz. a π delocalized system that can stack on the face of the quadruplex; a positive charge that can lie in the centre of the guanine-quartet; and positively charged terminal groups that can simultaneously bind in the grooves and loops of the quadruplex (Reed *et al*., 2006). It was found that the nickel-based complexes, Ni(II)-salphen {salphen =*N*,*N*'-bis(salicylidene)-3,4-diaminobenzoic acid}, could selectively bind and stabilise G-quadruplex DNA.

Kieltyka *et al.* (2007) showed that relatively simple platinum phenanthroimidazole complexes that bind duplex DNA by intercalation can also recognize and bind to quadruplex structures, and the observed binding constants to quadruplex DNA were nearly two orders of magnitude greater than for duplex DNA. Moreover, Reed and friends (2007) have also demonstrated that platinum(II) complexes could bind quadruplex structures with high affinity. Moreover, Kieltyka and co-workers (2008) designed and synthesised a square-shaped platinum complex, $[Pt(en)(4,4'\text{-dipyridyl})]_4(NO_3)_8$, that bound quadruplexes with high affinity and inhibited telomerase activity.

2.4 Topoisomerase I

Topoisomerases are isomerase enzymes that act on the topology of DNA. The main function for topoisomerases is unwinding and winding of DNA, in order for DNA to control the synthesis of proteins, and to facilitate DNA replication. The enzyme is necessary due to inherent problems caused by the DNA's double helix. In order to help overcome these problems caused by the double helix, topoisomerases bind to either single-stranded or double-stranded DNA and cut the phosphate backbone of the DNA. This intermediate break allows the DNA to be untangled or unwound, and, at the end of this process, the DNA is reconnected again (Champoux, 2001).

2.4.1 Topoisomerase I study

Generally, the process of semi-conservative DNA replication and accurate chromosome segregation requires that linking of the two interwined DNA strands be reduced to exactly zero, and unwinding of the two strands can generate positive supercoiling in front of the replication fork during DNA replication process. Topoisomerases are therefore required to rapidly relax the accumulated positive supercoils to allow progression of the replication fork. Furthermore, topoisomerases must also function to assure that the DNA strands are completely unlinked so that the replicated chromosomes can be segregated to the daughter cells. In addition, since the overall chemical composition and connectivity of the DNA does not change, the tangled and untangled DNAs are chemical isomers,

differing only in their global topology, thus their name (Wang, 1991; Champoux, 2001).

Fleishmann and co-workers (1984) observed that the topoisomerase I (topo I) became associated with actively transcribing heat shock genes but not with these loci when they were not being transcribed. In the studies of Chua *et al*. (2009), they had suggested that topo I was also associated with polymerase II transcription. These studies were based on an association of topo I with active sites of transcription.

Furthermore, Shykind *et al*. (1977) have further analyzed the role of the topo I in activating transcription, and have suggested that topo I likely functions during activation by enhancing the formation of an active TFIID-TFIIA (TF = transcription factor) complex on the promoter (Shykind *et al*., 1977). In addition, an analysis of factors that are required for high levels of transcription in response to a transcriptional activator uncovered another role for topo I in transcription. A group of researcher isolated a transcription factor from mammalian cells termed DR2 that was necessary for transcriptional activator stimulated transcription. Interestingly, DR2 was demonstrated to be identical with topo I (Kretzschmar *et al*., 1993).

The indolocarbazoles, typified by the antitumor antibiotic BE-13793C produced by a strain of *actinomycetes* (Kojiri *et al*., 1991) and its glycosylated

analog rebeccamycin isolated from a culture of *Saccharotrix aerocolonigenes* (Bush *et al*., 1987) have been found to be promising anticancer agents. 6-N-formylamino-12, 13-dihydro-1, 11-dihydroxy-13-(-D-glucopyranosyl)-5H-indolo [2, 3-a] pyrrolo-[3, 4-c] carbazole-5, 7-(6H)-dione or commonly known as NB-506 (Figure 2.11), has shown remarkable efficacy against a variety of human tumor xenografts, including lung and colon cancers, and metastatic cells. The exceptional antitumor activity of NB-506 is attributed to the capacity of the drug to intercalate into DNA and to inhibit topoisomerase I (Yoshinari *et al*., 1995). NB-506 has recently entered clinical trials (Yoshinari *et al*., 1995). NB-506 was able to convert topo I into a cell poison by trapping covalent DNA-topo I complexes, thereby enhancing the formation of persistent DNA breaks which led to the induction of cell death. In that respect, the mechanism of action of NB-506 closely resembles that of another series of antitumor agents recently introduced in the clinic, the camptothecins (Pommier *et al*., 1998). Other aspects of the mechanism of action of NB-506, including its interaction with nucleic acids, have not been characterized in any detail. Recently research from Bailly group reported that NB-506 intercalates preferentially into GC-rich sequences of B-DNA (Bailly *et al*., 1999). But apart from B-DNA, it is not known if NB-506 can interact with other nucleic acids structures such as single strand polymers, RNA, Z DNA, DNA triple helices, and tetraplexes (Ren *et al*., 2000).

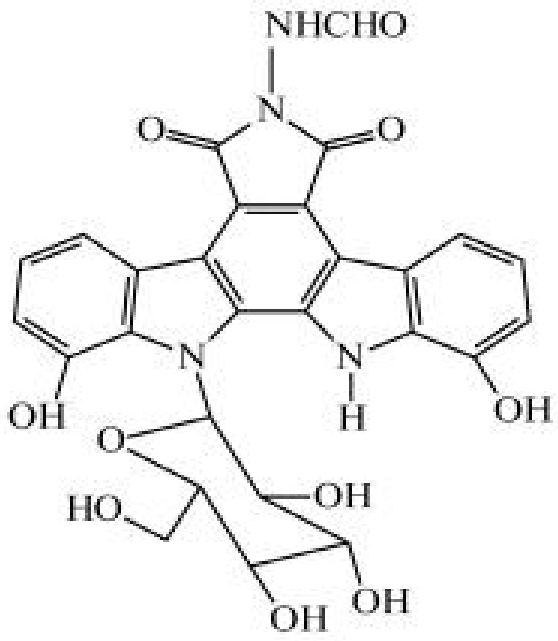


Figure 2.11: Structure of NB-506

(Arakawa *et al*., 1995)

Moreover, several groups of researchers have been reported that the indolocarbazole derivatives have shown complicated and distinct anti-tumor mechanisms including Topoisomerase I (Topo I) poisoning and inhibition of Protein Kinase C (PKC), Protein Kinase A (PKA), CDK1/cyclin B and CDK5/p25. Their different mechanisms of action are due to their structural diversity at some important substituted positions. For example, Rebeccamycin and NB-506 can form ternary complex with the DNA/Topo I duplex *via* intercalation in between the base pairs at the site of DNA cleavage (Yamashita *et al*., 1992; Anizon *et al*., 1997; Moreau *et al*., 1999; Bailly *et al*., 1999; Akinaga *et al*., 2000), while their analog termed Saturosporine, exhibits high levels of PKC

inhibition with no effect toward Topo I (Tamaoki *et al*., 1986; Nakano *et al*., 1987).

2.4.2 Topoisomerase I inhibitor

As we mentioned before, topoisomerase I ("one strand DNA cutter") is the enzyme that unwind and wind DNA, in order for DNA to control the synthesis of proteins, and to facilitate DNA replication. In order to help overcome these problems caused by the double helix, topoisomerases bind to either single-stranded or double-stranded DNA and induce transient cut of the phosphate backbone of the DNA. In recent years, topoisomerase I has become a focus in the development of anticancer drug. This is because some chemotherapy drugs work by interfering with topoisomerases in cancer cells. For example, topoisomerase I is the antigen recognized by Anti Scl-70 antibodies in scleroderma. These small molecule inhibitors act as efficient anti-bacterial and anti-cancer agents by preventing the natural ability of topoisomerase to create breaks in chromosomal DNA. Some chemotherapy drugs called topoisomerase inhibitors work by interfering with mammalian-type eukaryotic topoisomerases in cancer cells. This induces breaks in the DNA that ultimately lead to programmed cell death, or apoptosis (Champoux, 2001).

A DNA relaxation reaction assay that tested the catalytic activity of human topo I, was employed to investigate the effects of an asymmetric Tröger base containing the two well characterized DNA binding chromophores,

proflavine and phenanthroline. The compound 3-Amino-6*H*,16*H*-5,15-methano[1,5]-diazocino[2,3-*c*;6,7-*c'*]-[1,10]phenanthroline-acridine totally inhibits the relaxation of supercoiled plasmid DNA by topo I at a high drug concentration (>100 µM). The inhibition can be associated with the intercalative binding of the drug into DNA. Hence, this suggested that the drug can act as a poison for the enzyme, either by stimulating the cleavage step or by inhibiting the religation step in the catalytic cycle of the enzyme (Baldeyrou *et al*., 2002).

Zhao *et al.* (2004) published the first report of the topo I inhibitory activity of 2,4,6-trisubstituted pyridine compounds. A structure activity relationship study indicated that the 2-thienyl-4-furylpyridine skeleton was important for topo I inhibitory activity. The structure–activity relationship analysis revealed that the 2-thienyl-4-furylpyridine skeleton exhibited strong topo I inhibitory activity. In addition, McKnight *et al.* (2006) have shown the usefulness of the topo I inhibition assay in determining the relative DNA-binding affinities of homologous intercalators and the importance of substituent size during DNA binding. It was also used to provide evidence for the involvement of an intercalative binding mode for Pico Green and possibly SYBR Green which are two highly sensitive fluorescent dyes routinely used in a variety of double-stranded DNA assays and real-time PCR protocols.

In the context of the design and synthesis of minor groove binding and intercalating DNA ligands some new oligopyrrole carboxamides were synthesized.

These hybrid molecules (combilexins) possess a variable and conformatively flexible spacer at the N-terminal end. Incubation with increasing concentration of the various combilexins with supercoiled DNA plasmid in the presence of topo I revealed that the bis-pyrrole compounds efficiently intercalate with the co-planaric chromophore into the DNA helix as evidenced by the efficient formation of relaxed plasmid DNA using increasing concentrations of those compounds (David-Cordonnier *et al.*, 2007).

The mode of action of the antitumor drug, camptothecin, has been investigated by use of a partly double-stranded suicide DNA substrate which enables uncoupling of the cleavage and religation half-reactions of topo I (Kjeldsen *et al.*, 1992). The suicide DNA substrate contains a single topoisomerase I site at which sodium dodecyl sulfate; SDS cleavage is strongly enhanced by camptothecin on normal double-stranded DNA. Surprisingly, the results show that the religation reaction of topoisomerase I *per se* is strongly inhibited at this site compared to a site that is only marginally affected by camptothecin on double-stranded DNA. In addition, the research group also suggested that the inhibitory effect of camptothecin on cleavage is due to a general decrease in the non-covalent interaction of topoisomerase I with partly double-stranded suicide DNA substrates. Camptothecin is capable of inhibiting both the cleavage and religation steps of topo I, in a manner which is likely to depend on the stability of the noncovalent enzyme-DNA complex. Therefore, this

study directly demonstrates that camptothecin-mediated stability of a topoisomerase I-DNA complex is sequence-dependent (Kjeldsen *et al*., 1992).

Recently, the investigation of inorganic complexes with topoisomerase has gained prominence. A series of mononuclear and binuclear ruthenium(II) complexes of 1,10-phenanthroline, have been investigated by topoisomerization experiments. The results suggested that only binuclear phenanthrolyl ruthenium (II) complexes are able to intercalate between base pairs of the DNA coil in the presence of 4 mM $MgCl_2$ giving further evidence of their greater affinity towards DNA when compared to the corresponding mononuclear derivatives. The topoisomer profiles also confirmed the greater affinity induced by the triamino linker. Ruthenium(II) complexes of the bipyridyl series had no effect on the topoisomerization under the same conditions (Bouskila *et al*., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and solutions

In this study, all of the materials and chemicals were of analytical grade and purchased from commercial suppliers and used without any further purification unless otherwise stated. Plasmid DNA, pBR 322, 6X loading dye solution, ready-to-use Gene Ruler™ DNA ladder mix, restriction enzymes and human DNA topoisomerase I (topo I) were purchased from BioSyn Tech (Fermentas) and was stored at -20 ˚C. 30 % aqueous hydrogen peroxide (H_2O_2) from Merck (Germany), L-ascorbic acid from Acros Organics (USA), copper(II) chloride ($CuCl_2$), Tris, Phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium hydroxide (NaOH) and sodium chloride (NaCl) were from Fisher Scientific (UK), 1.5 % Agarose gel (Molecular Biology grade) from Promega (USA), synthetic oligonucleotides (PAGE purified) were ordered from 1st Base Sdn. Bhd. (Malaysia), Calf-Thymus (CT) DNA from Sigma, and Ethidium bromide from BioSyn Tech (Fermentas), were used as supplied. All solutions for DNA experiments were prepared with ultra-pure water from an Elga PURELAB ULTRA Bioscience water purification system with UV light accessory. All the copper(II) complexes, [Cu(phen)(D-ala)(H_2O)]Cl,

[Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3, were supplied by Assoc. Prof. Dr. Ng Chew Hee from University Tunku Abdul Rahman. These complexes have been fully characterized by chemistry students, Chin Lee Fang and Wang Wai San by means of FTIR spectroscopy, elemental analysis (CHN), UV-Visible spectroscopy, conductivity measurement, ESI-MS and X-ray crystallography.

3.2 Physical measurements

UV-visible spectroscopic measurement was carried out on a Lambda 35 UV/VIS spectrometer from Perkin Elmer (USA), and the fluorescence study was carried out with LS 55 Fluorescence Spectrometer from Perkin Elmer (USA). Fluorescence study of the interaction of metal(II) complexes with CT-DNA, ds(oligonucleotide) or G-quadruplex DNA was carried out with a 1.0 cm quartz cell. For studies on aqueous solution samples, distilled water was filled into the reference cell while for those on buffered solutions, the corresponding buffer was the reference material.

3.3 Preparation of copper(II) complexes solution

In this study, the copper(II) complexes were weighed in the range of 1.0 – 2.0 mg by using analytical balance. Then, the amounts of distilled water needed for top up purposes to prepare 10 mM of complexes were calculated (as calculation shown in the appendix). After that, the solutions were mixed

thoroughly by using vortex machine and ensure that all the complexes were dissolved completely in the distilled water. The complexes were diluted with buffer (or autoclaved ultra-pure water) to meet experiment requirements, by using $M_1V_1 = M_2V_2$.

The molecular weight for the copper(II) complexes is:

[Cu(phen)(D-ala)(H_2O)]Cl = 422.34 g/ mol

[Cu(phen)(L-ala)(H_2O)]Cl = 395.32 g/ mol

[Cu(phen)(D-threo)(H_2O)]Cl = 443.36 g/ mol

[Cu(phen)(L-threo)(H_2O)]Cl = 443.36 g/ mol

[Cu(phen)(D-threo)(H_2O)]NO_3 = 459.90 g/ mol

[Cu(phen)(L-threo)(H_2O)]NO_3 = 495.93 g/ mol

3.4 Preparation of 1.5 % (w/v) agarose gel

1.5 % agarose gel (100 ml) was prepared by adding 1.5 g of agarose gel powder and 100 ml of 1X TAE buffer in a 500 ml Schott bottle. After that, the mixture was heated and stirred evenly by using hot plate stirrer (Favorit, Malaysia), until all of the agarose gel powder was completely dissolved in the TAE buffer (pH 7.2). Then, the mixture was poured into the gel mold (Thermo) with comb and it was allowed to solidify at room temperature.

3.5 DNA cleavage studies (Nucleolytic properties)

Agarose gel electrophoresis experiments were carried out on supercoiled plasmid DNA pBR322 (4.4 kb) using a horizontal gel system. For the cleavage studies, each 20 μL sample consisted of the complex solution, DNA, and the required volume of additional buffer. All samples were incubated in the dark in an incubator at a temperature of 37 ºC. The reaction mixtures were prepared as follows: 1μl of 50 μM copper(II) complex or $CuCl_2$ salt was added to the mixture of 0.5 μl of supercoiled plasmid DNA pBR322 (0.5 μg/μl) and pH 7.2 Tris-NaCl buffer (5 mM tris; 50 mM NaCl) was added to give a total volume of 20 μl. The reactions were performed after incubating the reaction mixtures at 37 °C for 2 or 24 hours. 3 μl of 6x loading buffer was added to 20 μl of the given reaction mixture and each electrophoresis was performed at 80 V for 90 minutes in Tris-acetate-EDTA (TAE) buffer, pH 8.0, using 1.5 % agarose gel. After electrophoresis, the agarose gel was stained with ethidium bromide (EB) solution (0.5 μg/ml). For the oxidative or reductive cleavage studies, incubation of each sample was similarly carried out. The DNA cleavage profile was analyzed using 1.5 % agarose gel in a horizontal gel tank set with a running time of 2 hours, at a constant voltage of 80 V. Each reaction mixture consisted of 0.25 μg of DNA and pH 7.2 Tris-NaCl buffer unless otherwise mentioned. The resultant DNA bands after the electrophoresis step for each set of experiments were stained with ethidium bromide before being photographed under UV light using a Syngene Bio Imaging system and the digital image was viewed with Gene Flash software.

Similar experiments were carried out to study the effect of varying complex concentration, pH of buffer, the types of buffer and exogenous agent concentration. All final reaction mixtures were obtained by topping up with Tris-NaCl buffer pH 7.2.

3.6 DNA-binding studies

Stock solutions of calf thymus DNA (CT-DNA) were prepared by dissolving the DNA in buffer solution at 4 ºC, and the resultant homogeneous solutions were used within two days. The concentration of CT-DNA per nucleotide phosphate was calculated from the absorbance at 260 nm by using ε = 6400 M^{-1} cm^{-1}. The purity of the DNA was checked by monitoring the absorbance at 260 and 280 nm. PAGE grade of self-complimentary 12-mer oligonucleotides $(CG)_6$, $(AT)_6$, HPLC grade G-quadruplex 22-mer oligonucleotide 5‘-AGGGTTAGGGTTAGGGTTAGGG-3‘, and 17-mer complementary oligonucleotides 5‘-CCAGTTCGTAGTAACCC-3, 3‘-GGTCAAGCATCATTGGG-5‘ were annealed, to give the respective duplex and G-quadruplex, as specified by the supplier (from 1st Base Sdn. Bhd., Malaysia). The oligonucleotides and G-quadruplex were diluted with buffer (or autoclaved ultra-pure water) to meet experiment requirements, by using formula $M_1V_1 = M_2V_2$. Fluorescence (FL) emission spectra in the study of ethidium bromide quenching assay were recorded in the wavelength range 550–650 nm by exciting the respectively solutions with light at 545 nm. Excitation and emission slits were set at 10 nm. Solutions of DNA, and a series of copper(II) complexes were

prepared in TN buffer (5 mM Tris, 50 mM NaCl) at pH 7.2 unless specifically stated.

3.6.1 Ethidium bromide displacement assay

Ethidium bromide displacement assay were performed by measuring the emission of ethidium bromide bound to DNA which shows the enhanced emission intensity due to its intercalative binding to DNA. The competitive binding of the copper(II) complex to the DNA reduces the emission intensity of ethidium bromide (EB) with either the bound complex quenching the emission or a displacement of the bound EB from the bound to the free state. Fluorescence measurements were performed using a Perkin-Elmer LS55 photoluminescence spectrometer. All the fluorescence measurements were taken at excitation wavelength, λ_{ex} of 545 nm and emission wavelength, λ_{em} of 600 nm at room temperature. For determination of binding constant of copper(II) complex with CT-DNA, a TN buffer (5 mM Tris, 50 mM NaCl) at pH 7.2 was used. Prior to titration with the copper(II) complex, each 3 ml mixture of EB (0.32 μM) and CT-DNA (10 μM) was incubated for 24 hours to allow saturation of DNA with EB (Seng *et al.,* 2009). For the corresponding determination of binding constant with duplex deoxyoligonucleotides, the optimized conditions were based on previously reported procedure for high-throughput screening of DNA of short nucleotide sequences (Boger *et al.,* 2001). The ratio of duplex: EB is 1:2 and the TN buffer (pH 7.2) composition is 100 mM Tris and 100 mM NaCl. Prior to titration with copper(II) complex, each 3 ml mixture of EB (2 μM) and duplex oligo (1 μM)

was incubated for 24 hours to attain saturation. For both cases, the serial titration was completed by adding 1μl of increasing concentration of copper(II) complex from appropriate stock solutions to the series of 3 ml EB-DNA mixtures until the quenching of DNA-bound EB fluorescence exceeds 50 %. The final reaction mixtures were incubated for 2 hours before measurement of their fluorescence intensity. The fluorescence intensities were plotted against the complex concentration to yield a curve that showed the relative extent of quenching of DNA bound EB. The values of the apparent binding constant, K_{app}, of the copper(II) complex were calculated from the equation $K_{app,[complex]}$ [complex] = $K_{app,EB}$[EB] where $K_{app,EB}$ is the apparent binding constant of EB assumed to be 10^7 M^{-1}, $K_{app,complex}$ is the apparent binding constant of copper(II) complex, [EB] is the concentration of EB used and [complex] is the concentration of the copper(II) complex at 50% quenching (Rajendran et al., 2006).

3.6.2 Thiazole orange quenching assay

Thiazole orange (TO) quenching studies were conducted using oligo 22G 5'-$AG_3(T_2AG_3)_3$-3', and two complementary 17 nucleotide primers, 5'-CCAGTTCGTAGTAACCC-3' and 3'-GGTCAAGCATCATTGGG-5' (Monchaud *et al.,* 2006; Paritala *et al.*, 2009; Seng *et al.*, 2009). Previous researchers have shown that 22 G folds into a G-quadruplex while 17 bp adopts a duplex DNA structure. Binding to each oligonucleotide was determined by titrating the test copper(II) complex into a solution containing the oligonucleotide (1 μM) and TO (2 μM) in Tris-HCl buffer (pH 7.2). The fluorescence spectra

were taken and the area of the peak was determined using the instrument software, after each addition. Then the area was plotted as a function of copper(II) complex concentration and the concentration of copper(II) complex that reduced the area by 50 % was taken as the IC_{50} value. Binding constants were calculated assuming a simple competitive binding model (Stern-Volmer equation). The value of the apparent binding constant, K_{app}, of each copper(II) complex was calculated from the equation $K_{app,[complex]}$ [complex] = $K_{app,TO}$[TO] where $K_{app,TO}$ is the apparent binding constant of TO assumed to be 3 x 10^6 M^{-1}, $K_{app,complex}$ is the apparent binding constant of copper(II) complex, [TO] is the concentration of TO used and [complex] is the concentration of the copper(II) complex at 50% quenching (Monchaud *et al.*, 2006).

3.7 Restriction enzyme inhibition assay

The restriction enzyme inhibitory activity was determined by observing the resultant band of lambda (λ) DNA. Each reaction mixture contained 0.25 μg of λ DNA, 2 μl of 10x restriction enzyme reaction buffer, 50 μM of copper(II) complex or $CuCl_2$, 5 units of restriction enzyme and sterile deionized water. The total volume of each reaction is 20 μl. Firstly, λ DNA was incubated with copper(II) complex at 37 ^oC for 60 minutes and then restriction enzyme was added and the reaction mixture was incubated for another 2 hours at the same temperature. The reactions were terminated by the addition of 2 μl of 10 % SDS, and then followed by 3 μl of dye solution comprising 0.02 % bromophenol blue and 50 % glycerol. SDS is required to denature restriction enzyme, preventing

further functional enzymatic activity. The mixtures was applied to 2.0 % agarose gel and electrophoresed for 2 hours at 80 V with running buffer of Tris-acetate EDTA, TAE. The gel was stained, destained, and photographed under UV light using a Syngene Bio Imaging system and the digital image was viewed with Gene Flash software.

3.8 Human DNA topoisomerase I (topo I) inhibition assay

The human DNA topoisomerase I (topo I) inhibitory activity was determined by measuring the relaxation of supercoiled plasmid DNA pBR322. Each reaction mixture contained 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM Phenylmethylsulfonyl fluoride (PMSF), and 1 mM 2-mercaptoethanol, 0.25 μg plasmid DNA pBR322, 1 unit of topo I, and the test compound or copper(II) complex at a specified concentration. Total volume of each reaction mixture was 20 μl and these mixtures were prepared on ice. Upon enzyme addition, reaction mixtures were incubated at 37 ^{o}C for 30 minutes. The reactions were terminated by the addition of 2 μl of 10 % SDS, and then followed by 3 μl of dye solution comprising 0.02 % bromophenol blue and 50% glycerol. SDS is required to denature topoisomerase I, preventing further functional enzymatic activity. The mixtures was applied to 1.2 % agarose gel and electrophoresed for 5 hours at 33V with running buffer of Tris-acetate EDTA, TAE. The gel was stained, destained, and photographed under UV light using a Syngene Bio Imaging system and the digital image was viewed with Gene Flash software.

Same protocol was repeated in the topo I inhibition condition study. The only variation is the sequence in adding the main components (topo I, plasmid DNA pBR322, and copper(II) complex). Two conditions were studied in this assay. In the first condition, topo I was incubated with copper(II) complex at 37 ^{o}C for 30 minutes and then DNA was added and the reaction mixture was incubated for another 30 minutes at the same temperature. In the second condition, copper(II) complex and DNA were incubated for 30 minutes at 37 ^{o}C first before the addition of topo I. Then, the resultant reaction mixture was incubated for another 30 minutes at the same temperature before the application of the reaction termination step.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.0 Introduction

Based on the two main objectives of this research, the results are divided into two main parts, viz. nucleolytic study, and DNA binding study. In the nucleolytic study, various factors that might affect the nucleolytic efficiency of the copper(II) complexes were investigated by gel electrophoresis technique. The factors are concentration of copper(II) complexes, pH of buffer, type of buffer, incubation time, and the presence of exogenous agents viz. hydrogen peroxide (H_2O_2) and L-ascorbic acid.

For the DNA interaction study, binding constant and binding affinity of the copper(II) complexes for different types of DNA were investigated by photoluminescence techniques, i.e. ethidium bromide (EB) quenching assay and thiazole orange (TO) quenching assay. Different types of DNA that were employed in this experiment were calf thymus (CT) DNA, double stranded oligonucleotides with different sequences, and G-quadruplex DNA. The quenching assay were performed by measuring the emission of EB or TO bound

to DNA which shows the enhanced or reduced of emission intensity due to its intercalative binding to DNA in the presence of copper(II) complexes.

4.1 Nucleolytic study of chiral [Cu(phen)(aa)(H_2O)]X (X = Cl^-, NO_3^-)

In recent years, scientists have made great progress in research on the interaction between small organic molecules and nucleic acid, and in the cleavage of DNA at specific sites (Sigman *et al*., 1979; Rammo *et al*., 1996; Ng *et al*., 2006; Barceló-Oliver *et al*., 2007). Interestingly, many transition metal complexes were found to be efficient for sites-specific cleavage of DNA. The metal complexes usually contain three components: a binding group to recognize the special sequence of nucleic acid, a chemically active group to cleave the nucleic acid and a spacer to connect the former two parts (Karlin *et al*., 2002; Zhang *et al*., 2004).

Transition metal complexes capable of cleaving DNA are of importance for their potential use as new structural probes or artificial nucleases in nucleic acids chemistry and as therapeutic agents. Especially for the complexes showing induction of DNA cleavage in the absence of exogenous agents are of particular interest as they can be made effective in highly targeted therapeutic applications (Reddy *et. al.*, 2004).

Hence, this nucleolytic study involving chelated chiral amino acid is relatively important and essential in investigating the factors that affect the nuclease activity of a chiral metal complex.

4.1.1 Nucleolytic study without exogenous agent

This nucleolytic study was performed by using double stranded plasmid DNA, pBR322 with agarose gel electrophoresis technique. The pBR322 is a commonly used plasmid cloning vector in E. *coli* (Bolivar *et. al.*, 1977). The molecule is a double-stranded circular DNA and 4361 base pairs in length. The plasmid has unique restriction sites for more than forty restriction enzymes (Watson, 1988).

Plasmid pBR322 DNA is useful to study the DNA cleavage activity of an unknown compound due to its high availability, affordable price and ease in data analysis. The consequence of cleavage of circular pBR322 is relaxation of the supercoiled form of DNA into nicked (relaxed open circular) form and/or linear form (Figure 4.10). Nicked form of DNA will be formed if only one strand from the double strands has been cleaved. Linear form of DNA will be formed if both strands of the double stranded pBR322 have been cleaved. Different DNA forms can form as a result of different extent of cleavage of the plasmid DNA and these can be visualized on the agarose gel. The supercoiled form of DNA has the fastest migration rate, linear form has intermediate migration rate, and nicked form of DNA moves the slowest when submitted to agarose gel electrophoresis (Czene *et. al.*, 1997).

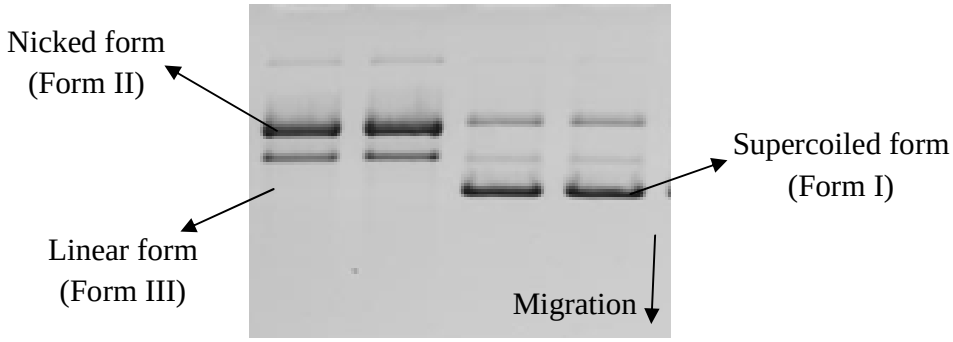


Figure 4.10: Diagram of agarose gel electrophoresis shown the plasmid is separated according to its topology.

Agarose gel electrophoresis is the easiest and the most common way of separating and analyzing DNA. The purpose of this technique might be to look at the DNA, to quantify it or to isolate a particular band. Agarose gels are easily cast and handled compared to other matrices and nucleic acids are not chemically altered during electrophoresis. Samples are also easily recovered. In addition, nucleic acid molecules can be easily separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. Next, the DNA is visualized in the gel by addition of ethidium bromide (EB). EB binds strongly to DNA by intercalating between the bases and the bound EB emits fluorescence strongly upon UV illumination (Sambrook and Russel, 2001).

Lastly, by observing the plasmid DNA cleavage pattern on electrophoresis gel image, the nucleolytic efficiency of a series metal complex can be qualitatively compared.

4.1.1.1 The effect of complex concentration on DNA cleavage activity

The effect of concentration of various copper(II) complexes was investigated over the range 10 μM – 2 mM in Tris-NaCl buffer (TN buffer), (5 mM Tris; 50 mM NaCl) at pH 7.2, with incubation time of 24 hours and an incubation temperature of 37 ^{o}C. In this section, a series of copper(II) complexes, [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 were used to investigate the relative contribution of the type of ligand in moderating the nucleolytic efficiency and to study what kind of ligand will give rise to more efficient nucleolytic metal complex.

Interestingly, not all copper(II) complexes possessed the same nucleolytic efficiency. It can be seen that the induction of cleavage happened starting from 10 μM for [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, and [Cu(phen)(L-threo)(H_2O)]Cl (Figure 4.11 (a – d), L4). All four of these copper(II) complexes were able to convert some supercoiled DNA into nicked DNA with the increasing complex concentration (Figure 4.11 (a – d), L4 – 9). In addition, the nucleolytic efficiency of [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 seems to be very low and very similar to each other. With the increase of complex concentration from 10 μM to 2 mM, both [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 were only able to convert a very small amount of supercoiled DNA into nicked form of DNA (Figure 4.12, L4 – 9; Figure 4.13, L5 – 10).

Complex concentration can be an inhibitory or enhancing factor in DNA cleavage activity for a metal complex. However, complex concentration seems to be one of the limiting factors in DNA cleavage activity in this study. Although the DNA cleavage activity was carried out with increasing complex concentration, but there was no further increase in DNA cleavage efficiency while the complex concentration was increased. When the complex concentration was increased from 10 μM – 2 mM, the amount of cleaved DNA seems to remain constant after a certain limiting concentration of copper(II) complex is reached (Figure 4.11 (a – d), L4 – 9; Figure 4.12, L4 – 9; Figure 4.13, L5 – 10).

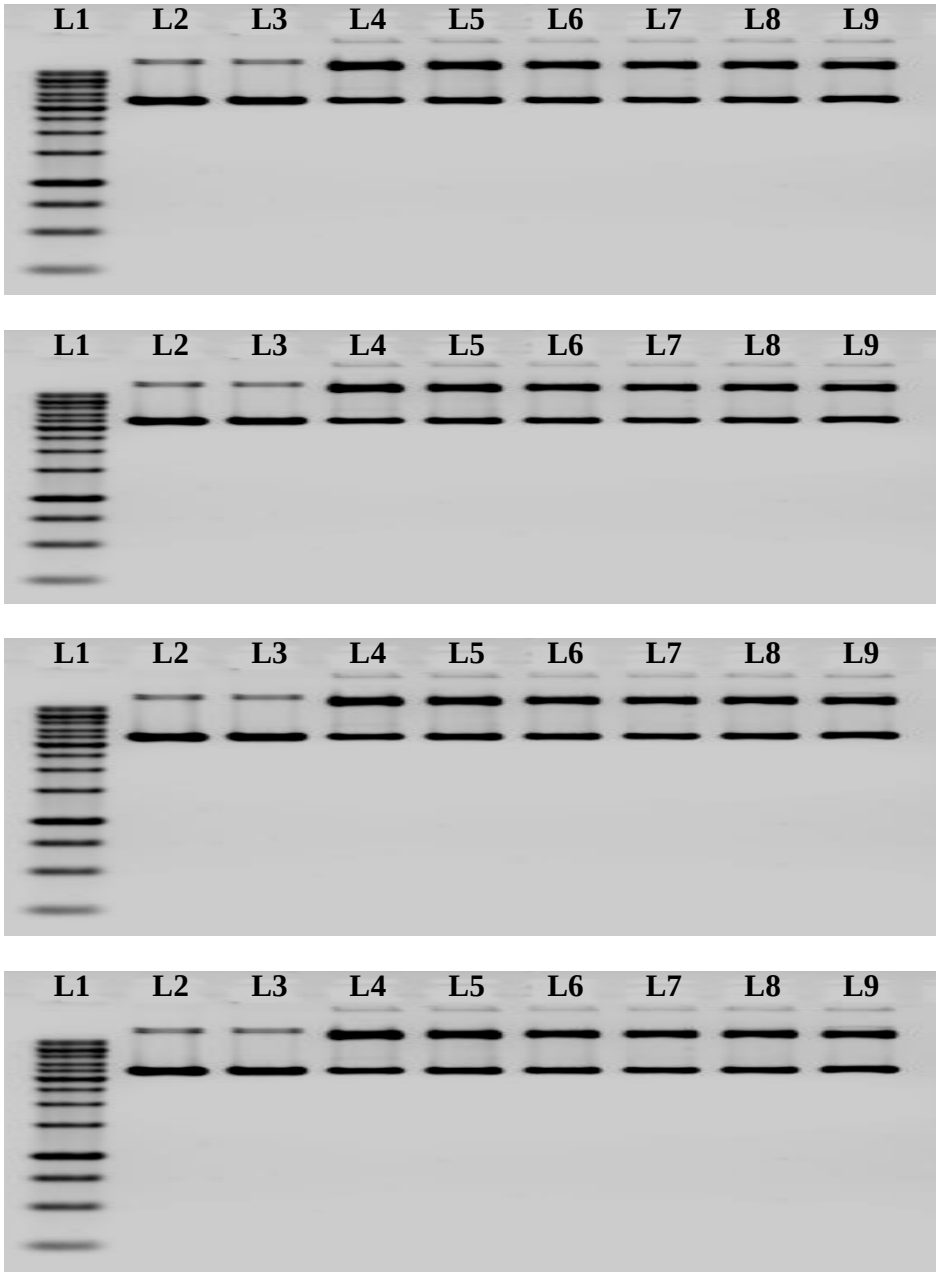


Figure 4.11: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of (a), [Cu(phen)(D-ala)(H_2O)]Cl; (b), [Cu(phen)(L-ala)(H_2O)]Cl; (c), [Cu(phen)(D-threo)(H_2O)]Cl; (d), [Cu(phen)(L-threo)(H_2O)]Cl in 5 mM TN buffer pH 7.2 at various concentration (10 μM – 2 mM) for 24 hours at 37ºC. L1, Gene Ruler 1Kb DNA Ladder; L2, Untreated DNA Control; L3, DNA + 2 mM $CuCl_2$; L4, DNA + 10 μM complex; L5, DNA + 50 μM complex; L6, DNA + 200 μM complex; L7, DNA + 500 μM complex; L8, DNA + 1 mM complex; L9, DNA + 2 mM complex.

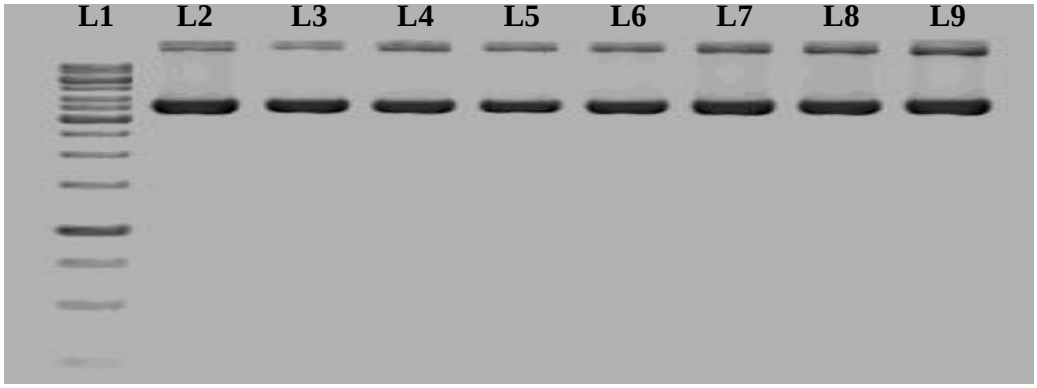


Figure 4.12: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of [Cu(phen)(D-threo)(H_2O)]NO_3 in 5 mM TN buffer pH 7.2 at various concentration (10 μM – 2 mM) for 24 hours at 37ºC. L1, Gene Ruler 1 Kb DNA Ladder; L2, Untreated DNA Control; L3, DNA + 2 mM $CuCl_2$; L4, DNA + 10 μM complex; L5, DNA + 50 μM complex; L6, DNA + 200 μM complex; L7, DNA + 500 μM complex; L8, DNA + 1 mM complex; L9, DNA + 2 mM complex.

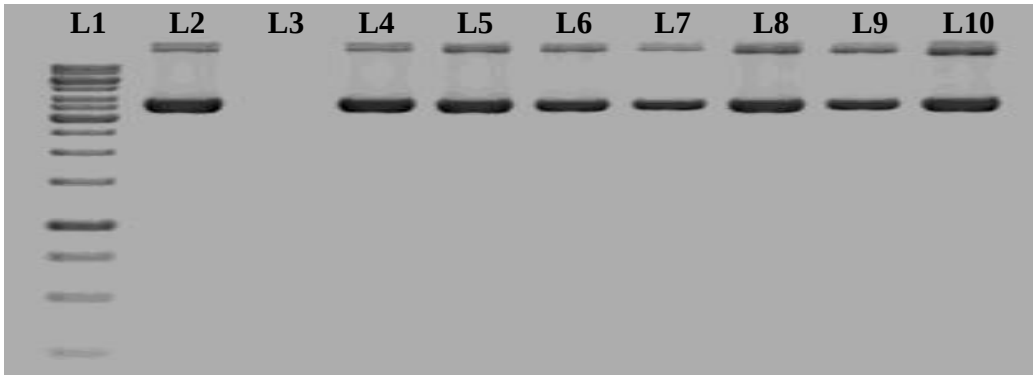


Figure 4.13: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of [Cu(phen)(L-threo)(H_2O)]NO_3 in 5 mM TN buffer pH 7.2 at various concentration (10 μM – 2 mM) for 24 hours at 37ºC. L1, Gene Ruler 1 Kb DNA Ladder; L2, Untreated DNA Control (0.5 μg/μL); L3, Empty; L4, DNA + 2 mM $CuCl_2$; L5, DNA + 10 μM complex; L6, DNA + 50 μM complex; L7, DNA + 200 μM complex; L8, DNA + 500 μM complex; L9, DNA + 1 mM complex; L10, DNA + 2 mM complex.

Seng *et al*., 2008 suggested that with the increase of concentration of [M(phen)(edda)], (M = Cu, Co, Zn or Ni), there is obvious increase of the induction of DNA cleavage of plasmid DNA, pBR 322. However, although DNA cleavage increases with concentration for both Cu and Co complexes, there seems to be a limiting concentration, beyond which there is no further increase in cleavage, and this suggested that the binding between complex and DNA was site-specific and site-selective. In contrast, DNA-binding and cleavage studies of novel copper(II) complex with L-phenylalaninate and 1,4,8,9-tetra-aza-triphenylene ligands studied by Li *et al.*, 2005, have shown that the supercoiled plasmid DNA was gradually converted into nicked or linear form of DNA counterparts with the increase of complex concentration.

Liang *et al.*, 2004, reported that the nickel(II) complex of 1,4,7-triazecan-9-ol at 50 μM could not cleave pBR322 in the absence of exogenous agent but it could completely convert supercoiled DNA into nicked and linear DNA when the complex concentration was increased to 500 μM. Nevertheless, Song *et al*., 2006 also stated that when the complex concentration of $[Ni(RA)_2(H_2O)_2]\cdot H_2O$ (RA = retinoic acid) was $\geq$ 0.02 mM, the complex could almost promote the complete conversion of DNA from supercoiled form of DNA to linear form of DNA after 70 minutes incubation in Tris-HCl buffer (pH 7.44) at 37°C.

4.1.1.2 The effect of types of buffer on DNA cleavage activity

Buffers are ubiquitous components in most *in vitro* reaction system, due to the reason that they are used to maintain the pH of the solution constant while other components in the reaction mixture are varied (Sambrook *et al.*, 1987). DNA cleaving ability has been investigated in three different types of buffer at 37 °C using 50 µM of each complex. The reaction mixtures were incubated for 24 hours in the absence of any oxidizing or reducing agents. Three different types of buffer that were chosen are HN, PN, and TN buffers at pH 7.2. For this variable, pH 7.2 was chosen for the pH of all buffers as it can be considered as physiological pH.

- Buffer 1 = Hepes-NaCl (HN) (Hepes = 20 mM; NaCl = 30 mM)
- Buffer 2 = Phosphate-NaCl (PN) (Phosphate = 20 mM; NaCl = 30 mM)
- Buffer 3 = Tris-NaCl (TN) (Tris = 20 mM; NaCl = 30 mM)

Figures 4.14 - 4.16 show the electrophoretic results of the different types of buffer on nucleolytic efficiency of the copper(II) complexes. Plasmid DNA alone did not undergo any cleavage or degradation after incubated for 24 hours in all these three buffers (Figure 4.14, L2 – 4). On the other hand, copper salt, $CuCl_2$ able to convert supercoiled DNA to nicked and linear form of DNA in Hepes buffer, but it only able to convert some supercoiled DNA to nicked form of DNA in phosphate and Tris buffers (Figure 4.14, L6 – 8).

As can be seen from the results, 50 μM copper(II) complexes, [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 were able to convert supercoiled DNA into nicked and linear form of DNA in Hepes buffer (Figure 4.15, L2, L6, L10 & L14; Figure 4.16, L6, L10). Comparatively, extent of DNA cleavage of the above listed complexes in both phosphate and Tris buffers were found to be lesser compared to Hepes buffer. For complexes [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, and [Cu(phen)(L-threo)(H_2O)]Cl, the nucleolytic efficiency was found to be similar in phosphate and Tris buffer, as no distinct difference in the amount of cleaved DNA was observed between these two buffers (Figure 4.15, L3 – 4, L7 – 8, L15 – 16). However, for complexes [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3, the nucleolytic efficiency was found to be stronger in phosphate buffer compared to Tris buffer as almost all of the supercoiled DNA were converted into nicked DNA after 24 hours incubation time (Figure 4.15, L11 – 12; Figure 4.16, L7 – 8, L11 – 12).

The results showed that Hepes buffer was the most suitable buffer for the study of nucleolytic efficiency. Most of the copper(II) complexes studied shows better cleavage ability in Hepes buffer, the supercoiled DNA being cleaved to yield nicked and linear form DNA.

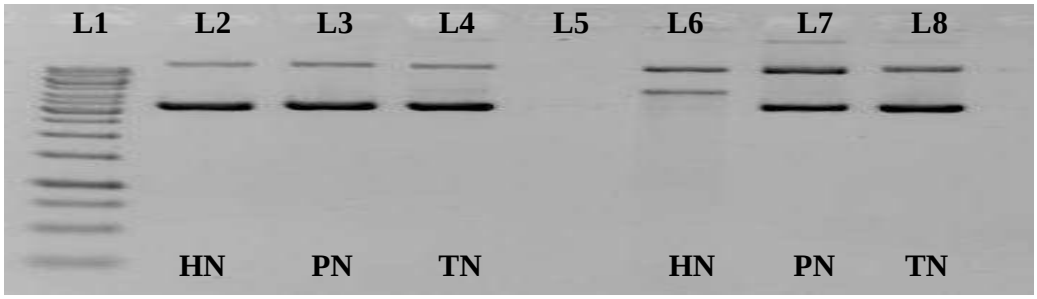


Figure 4.14: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence or absence of 50 μM $CuCl_2$ at pH 7.2 in various buffers for 24 hours at 37ºC. L1, Gene Ruler 1 Kb DNA Ladder; L2 – 4, Untreated DNA control; L5, Empty; L6 – 8, DNA + 50 μM $CuCl_2$.

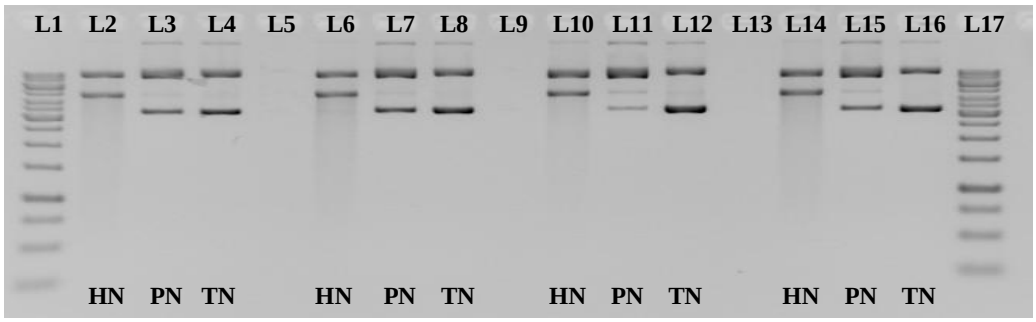


Figure 4.15: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of 50 μM complex at pH 7.2 in various buffers for 24 hours at 37ºC. L2 – 4, 50 μM [Cu(phen)(D-ala)(H_2O)]Cl; L6 – 8, 50 μM [Cu(phen)(L-ala)(H_2O)]Cl; L10 – 12, 50 μM [Cu(phen)(D-threo)(H_2O)]Cl; L14 – 16, 50 μM [Cu(phen)(L-threo)(H_2O)]Cl; L1 & 17, Gene Ruler 1 Kb DNA Ladder; L5, 9 & 13, Empty.

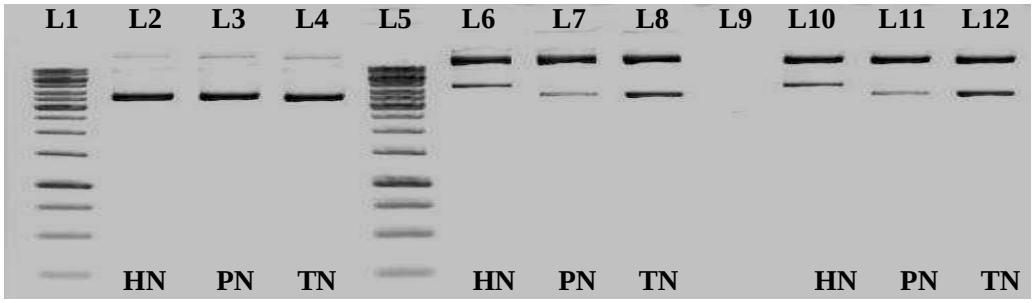


Figure 4.16: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of 50 μM complex at pH 7.2 in various buffers for 24 hours at 37ºC. L2

– 4, Untreated DNA control; L6 – 8, DNA + 50 µM [Cu(phen)(D-threo)(H_2O)]NO_3; L10 – 12, DNA + 50 µM [Cu(phen)(L-threo)(H_2O)]NO_3.

In the study of nucleolytic properties, sometime this tris(hydroxymethyl)aminomethane, or commonly known as Tris, will be the inhibitory factor for nucleolytic efficiency in Tris-type of buffers if its concentration is sufficient high. In addition, buffer effect can be due to their relative efficiency in scavenging radical species which are responsible for DNA cleavage (Hicks and Gebicki, 1986). However, phosphate buffer can be a problem for cationic metal complexes due to precipitation or complexation (Wang and Sayre, 1989).

Hepes was previously shown to act as a reductant to reduce copper(II) to copper(I) in the presence of ligands that stabilize copper(I). This may lead to the generation of free radicals which was responsible for DNA cleavage. Besides that, Hepes is an undeniable, but weakly competitive chelator of copper(II), and it does not seem to bind to metal complexes easily due to its bulkiness (Sokolowska *et al*., 2005).

Furthermore, Wenner and Bloomfield, 1999, also reported that DNA–buffer interactions influenced the rate of cleavage of plasmid pBR322 by the restriction enzyme *Eco*RV. This effect was attributed to differences in the binding of the enzyme to the DNA in various neutral pH buffers. The choice of buffer and the concentration of buffer component(s) is thus significant when ensuring higher

nucleolytic activity or when comparing the nucleolytic efficiency of homologous or non-homologous metal complexes.

4.1.1.3 The effect of pH of buffer on DNA cleavage activity

Human body pH is very important because pH controls the speed of human body's biochemical reactions. It does this by controlling the speed of enzyme activity as well as the speed that electricity moves through our body. The pH of different cellular compartments, body fluids, and organs is usually tightly regulated in a process called acid-base homeostasis. Acid-base homeostasis is a process that concerns the proper balance between acids and bases, or more accurately, the pH. The body is very sensitive to its pH level, if the pH falls outside the acceptable range, proteins might be denatured and digested, enzymes lose their ability to function, and this might be fatal. Hence, there exist some strong mechanisms to maintain it. The pH of blood is usually slightly basic with a value of pH 7.4. This value is often referred to as physiological pH in biology and medicine (Wu *et al*., 2008; McMillan and Cameron, 2005).

In this section, the effect of pH on DNA cleaving activity has been investigated with supercoiled plasmid DNA pBR322, in the absence of any exogenous agent. The plasmid DNA were incubated with 50 µM of each copper(II) complex at various pH values of the TN buffer (in the range of 6.2 – 8.2) at 37 °C for 24 hours.

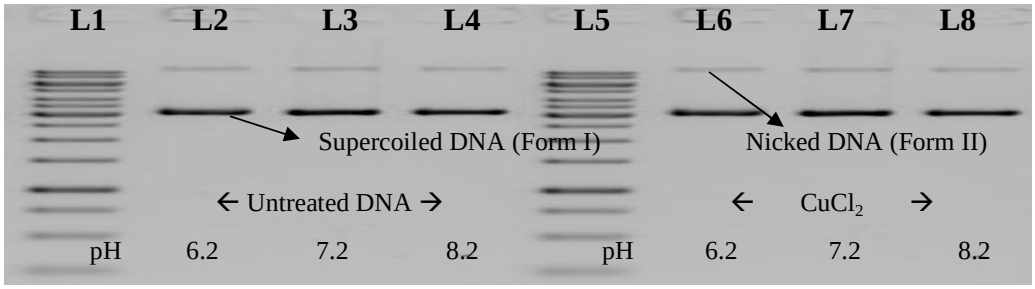
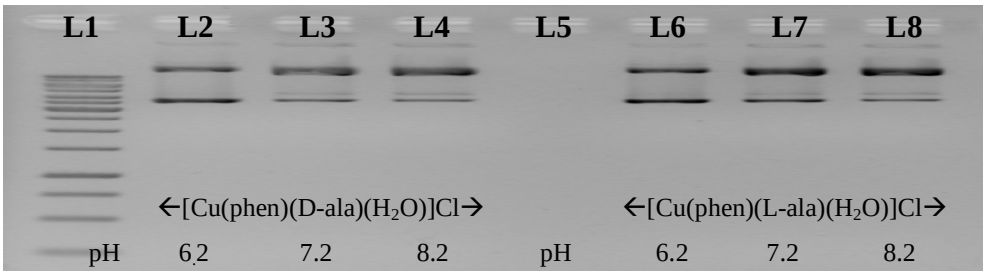
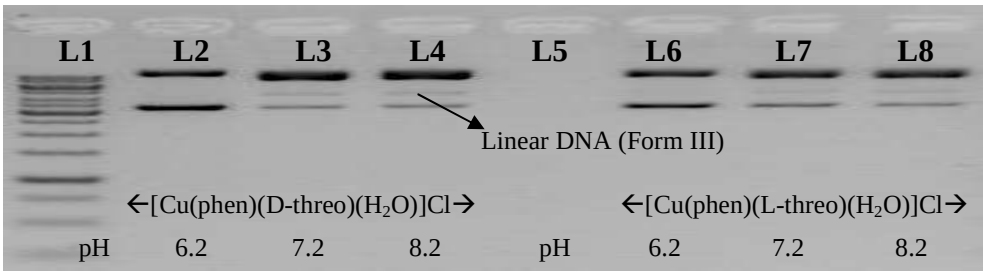


Figure 4.17 : Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence or absence of 50 μM $CuCl_2$ in TN buffer at various pH values for 24 hours at 37ºC. L2 – 4, Untreated DNA control; L5, Empty; L6 – 8, DNA + 50 μM $CuCl_2$; L1 & 5, Gene Ruler 1 Kb DNA Ladder.

(a)



(b)



(c)

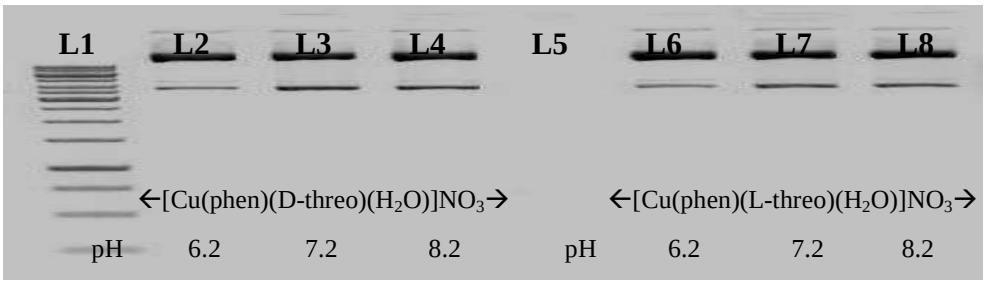


Figure 4.18 : Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of 50 μM (a), [Cu(phen)(D-ala)(H_2O)]Cl/ [Cu(phen)(L-ala)(H_2O)]Cl; (b), [Cu(phen)(D-threo)(H_2O)]Cl/ [Cu(phen)(L-threo)(H_2O)]Cl; (c), [Cu(phen)(D-threo)(H_2O)]NO_3/ [Cu(phen)(L-threo)(H_2O)]NO_3 in TN buffer at various pH values for 24 hours at 37ºC. L1, Gene Ruler 1 Kb DNA Ladder; L2 – 4, DNA + 50 μM complex; L5, Empty; L6 – 8, DNA + 50 μM complex.

The circular plasmid pBR322 alone (Figure 4.17, L2 – 4) did not undergo any DNA cleavage or degradation even in the presence of 50 µM $CuCl_2$ salt (Figure 4.17, L6 – 8). All of the plasmid DNA remains in supercoiled form (Form I) when incubated at 37 °C for 24 hours in TN buffer at pH 6.2, 7.2 and 8.2.

An increase of pH value of the TN buffer from 6.2 to 8.2 slightly enhanced DNA cleavage of complexes [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, and [Cu(phen)(L-threo)(H_2O)]Cl (Figure 4.18 (a – b); L2 – 4, L6 – 8). The nucleolytic efficiency of above listed complexes was found to be greater in TN buffer at pH 8.2 compared to pH 6.2 and pH 7.2, as they were able to convert almost all the supercoiled DNA into nicked DNA (Form II) and small amount of linear DNA (Form III) (Figure 4.18 (a – b), L4 & 8). Surprisingly, DNA cleavage by [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 complexes were found to be slightly better at pH 6.2 in TN buffer (Figure 4.18 (c), L2 & 6). These two complexes were able to convert almost all the supercoiled DNA into nicked DNA in this slightly acidic pH. However, the nucleolytic properties of [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 are suggested to be weaker compared to other four complexes, as they were only able to convert the supercoiled form of DNA into nicked form of DNA. There is no linear form of DNA induced at any pH of the TN buffer. Moreover, for each pairs of D- and L-enantiomer, i.e., [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-

threo)(H_2O)]NO_3, the cleavage affinity over the pH range from 6.2 – 8.2 were very similar to each other, it is very difficult to differentiate whether D- or L-enantiomer complexes show better cleavage affinity at the particular pH.

In 2006, Song *et al.* reported that changing pH value had only slight influence on DNA cleavage by $[Ni(RA)_2(H_2O)_2]\cdot H_2O$ after incubation for 80 minutes at 37 °C in 10 mM Tris–HCl buffer with 6.2 mM NaCl. Rittich *et al.*, 2004, also suggested that the DNA cleavage studies tested in the presence of free lanthanide cations—Eu^{3+}, La^{3+}, Nd^{3+}, Pr^{3+} and Gd^{3+} showed that pH influence was not important in tested range (pH 7.0 – pH 8.0) as it only shown no/ slight differences in catalytic activity. In addition, the studies of Wood and Lee, 2004, conclusively demonstrated that there is very little amount of double stranded DNA denatured by Zn(II) or Ni(II) during metal ion-DNA interaction at lower pH, but the amount of denatured DNA suddenly increased at pH 8.5. Hence, pH can affect the interaction between metal ion or metal complex and DNA.

Early in 1997, Czene *et al.* suggested that pH will affect the induction of DNA cleavage in permeabilized human fibroblasts. Acidic pH-induced strand breakage (pH-dependent DNA breakage; PDDB) in permeabilized human fibroblasts, with a maximum effect at pH 6.25, and activation of unknown endonucleases was suggested to be the factor responsible. Besides that, the anti-proliferative property of a ruthenium(II) complex of a thiosemicarbazone, tested

on ovarian carcinoma, has been reported to be about six times higher at pH 6.0 than at pH 7.4 (Grguric-Sipka *et al.*, 2007).

Moreover, 1, 7-dimethyl-1,4,7,10-tetra-azacyclododecane could hydrolyze double stranded DNA under physiological conditions (37 °C, pH 7.2) (Wan *et al.*, 2006). Zhao *et al.*, 2007, claimed that [2,9-tetramethyl-4,7-diaza-4-(4'-methyl-β-piperidinylethyl)-decane-2,9-dithiol], could be most effective for DNA cleavage, under physiological condition (pH 7.0). However, Frey *et al.*, 1996, reported that the nucleolytic efficiency of $[Cu_3(L)(NO_3)_2(H_2O)_3](NO_3)_4 \cdot 5H_2O$ were found to be pH-dependent. It cleaves DNA slowly in the absence of H_2O_2 and the cleavage abilities are more efficient at pH 9 or 10, rather than pH 7 or 8. All these evidences clearly show that most of the chemical reactions as well as nucleolytic efficiency are pH-dependent. The type of coordinated ligands used in the current study may be responsible for allowing pH to affect their nucleolytic efficiency. However, the effect of pH on the nucleolytic efficiency of the $[Cu(phen)(aa)(H_2O)]X$ is not great.

4.1.1.4 The effect of incubation time on DNA cleavage activity

The effect of varying the incubation time of the various copper(II) complexes was investigated with plasmid DNA, pBR322, in the presence of 50 μM of copper(II) complex in TN buffer at pH 7.2. The reaction mixtures were incubated at 37 ºC for 24, 48, and 72 hours, respectively. The pBR322 DNA alone (Figure 4.19, L2 – 4) did not undergo any cleavage or degradation over the incubation period from 24 to 72 hours as the DNA pattern (mainly of the supercoiled DNA band and small amount of nicked DNA band) remained unchanged with time. However, for the metal salt, $CuCl_2$, there was some increase of nucleolytic efficiency with the increase of incubation time. After 72 hours of incubation period, most of the supercoiled DNA has been cleaved into nicked DNA, and a small amount of DNA has been converted into linear DNA (Figure 4.19, L6 – 8).

The nucleolytic efficiency of [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl are very similar to each other (Figure 4.20, L2 – 4, L6 – 8). It seems that changing the subsidiary ligands from D-ala to L-ala in [Cu(phen)(aa)(H_2O)]Cl does not affect the nucleolytic efficiency of the copper(II) complexes. After 24 hours of incubation time, a large amount of supercoiled DNA has been converted into nicked form of DNA. With the increase of incubation period from 24 to 72 hours, there was a distinct increase of nucleolytic efficiency of the above listed complexes. Both the complexes successfully converted all

supercoiled DNA into nicked and linear form DNA after incubated for 72 hours (Figure 4.20, L4 & 8).

Interestingly, after 48 hours incubation period, [Cu(phen)(D-threo)(H_2O)]Cl converted almost all of the supercoiled DNA into nicked and linear from of DNA, with only a very faint band of supercoiled DNA can be seen as shown in L11, Figure 4.20. This complex was able to convert all supercoiled DNA into nicked and linear form DNA after 72 hours incubation time. However, [Cu(phen)(L-threo)(H_2O)]Cl could only convert most of the supercoiled DNA into nicked and linear form DNA after incubation period of 72 hours. There was still a faint band of supercoiled DNA can be observed in the gel image (Figure 4.20, L16). From here, we concluded that [Cu(phen)(D-threo)(H_2O)]Cl is slightly more effective nucleolytic agent compared to [Cu(phen)(L-threo)(H_2O)]Cl in this study.

However, [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 possessed similar nucleolytic efficiency and they could only converted supercoiled DNA into nicked DNA after incubated for 72 hours (Figure 4.21, L2 – 4, L6 – 8). A small amount of supercoiled DNA still remains uncleared. Both of these complexes are found to be the most inefficient nucleolytic agent in this study, as other four complexes able to convert all supercoiled DNA into nicked and linear DNA after incubated for 72 hours. From the comparative study, the order of nucleolytic efficiency of these six complexes is [Cu(phen)(D-

threo)(H_2O)]Cl > [Cu(phen)(D-ala)(H_2O)]Cl ≈ [Cu(phen)(L-ala)(H_2O)]Cl > [Cu(phen)(L-threo)(H_2O)]Cl > [Cu(phen)(D-threo)(H_2O)]NO_3 ≈ [Cu(phen)(L-threo)(H_2O)]NO_3.

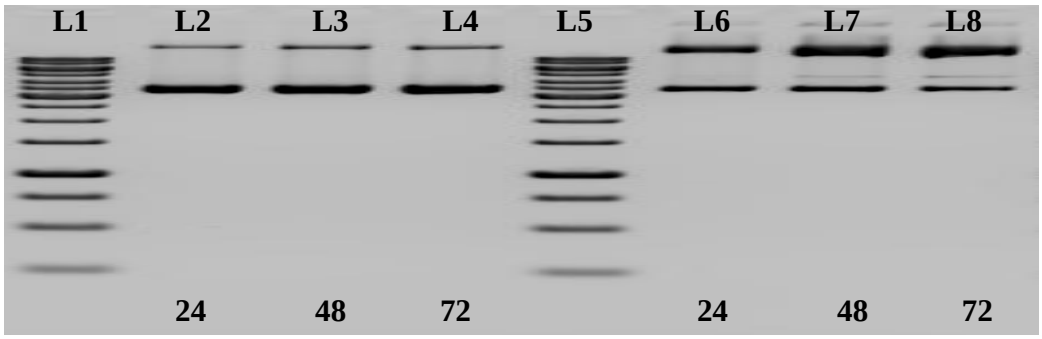


Figure 4.19: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence or absence of 50 μM $CuCl_2$ in TN buffer at pH 7.2 at various incubation times at 37ºC. L2 – 4, Untreated DNA control; L6 – 8, DNA + 50 μM $CuCl_2$; L1 & 5, Gene Ruler 1 Kb DNA Ladder.

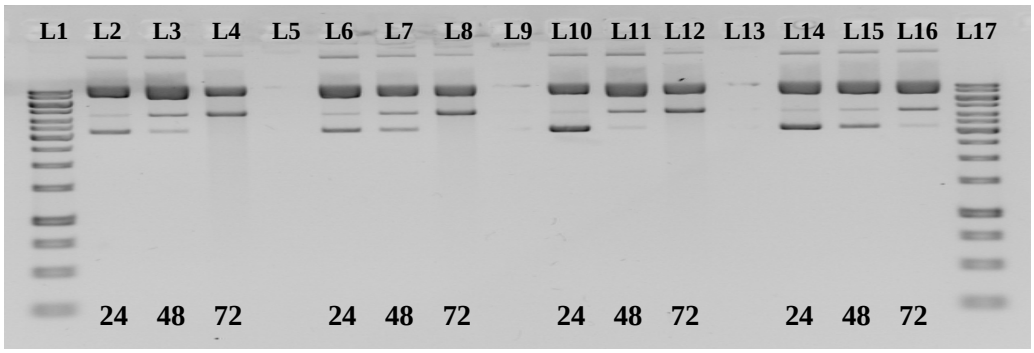


Figure 4.20: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of 50 μM complex in TN buffer at pH 7.2 at various incubation times at 37ºC. L2 – 4, DNA + 50 μM [Cu(phen)(D-ala)(H_2O)]Cl; L6 – 8, DNA + 50 μM [Cu(phen)(L-ala)(H_2O)]Cl; L10 – 12, DNA + 50 μM [Cu(phen)(D-threo)(H_2O)]Cl; L14 – 16, DNA + 50 μM [Cu(phen)(L-threo)(H_2O)]Cl; L1 & 17, Gene Ruler 1 Kb DNA Ladder; L5, 9 & 13, Empty.

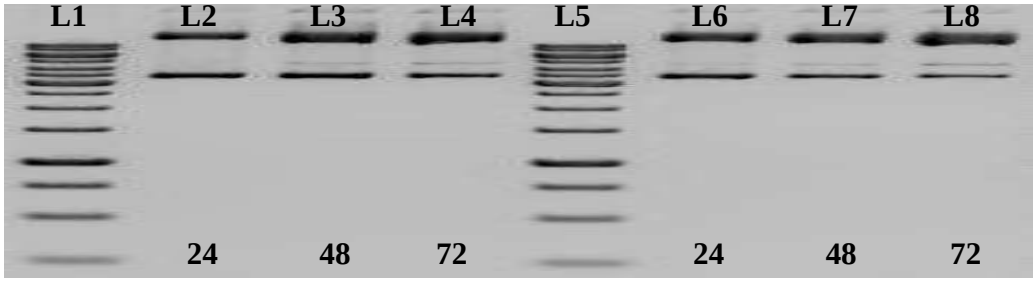


Figure 4.21: Electrophoresis results of incubating pBR322 (0.5 μg/μL) in the presence of 50 μM complex in TN buffer at pH 7.2 at various incubation times at 37ºC. L2 – 4, DNA + 50 μM [Cu(phen)(D-threo)(H_2O)]NO_3; L6 – 8, DNA + 50 μM [Cu(phen)(L-threo)(H_2O)]NO_3; L1 & 5, Gene Ruler 1Kb DNA Ladder.

The time course study of DNA cleavage by $[Ni(RA)_2(H_2O)_2]\cdot H_2O$ showed that with the increase of reaction time, the supercoiled DNA diminished gradually and amount of linearized DNA increased. After 40 min, the linearized DNA began to appear, and the supercoiled DNA was barely observable (Song *et al.*, 2006). Interestingly, at 72 hours incubation the nicking of the DNA by the [M(phen)(edda)] (M = Co, Ni and Zn) complexes was more pronounced compared to those incubated for 24 and 48 hours. The nucleolytic efficiency of the [M(phen)(edda)] complexes was found to depend on both the incubation time and the nature of metal ion (Seng *et al.*, 2008).

Dixon *et al.*, 1996, also reported that incubation time was one of the factors that affected the amount of DNA cleavage. Cobalt complexes, $[(en)_2Co(OH)(OH_2)]^{2+}$, $[(cyclen)_2Co(OH)(OH_2)]^{2+}$, and $[(tamen)_2Co(OH)(OH_2)]^{2+}$ were found to promote hydrolysis of pUC19 plasmid DNA. With the increase of incubation period from 0 minute to 6 hours, the complexes slowly converted the supercoiled DNA into nicked and linear form of DNA.

4.1.2 Nucleolytic study with exogenous agent

In previous section, we had investigated the effect of incubation conditions, i.e., complex concentration, pH of buffer and incubation time, towards nucleolytic property of the ternary copper(II) complexes. In this section, the effect of exogenous agent on the DNA cleavage of the ternary copper(II) complexes was studied. The exogenous agents are hydrogen peroxide (H_2O_2) and L-ascorbic acid (AA). The former is an oxidizing agent while the latter is an anti-oxidant and a reducing agent.

4.1.2.1 Nucleolytic study in the presence of hydrogen peroxide, H_2O_2

Hydrogen peroxide, H_2O_2, which is an oxidizing agent that was used in this study, can be converted into hydroxyl radicals in the presence of transition metal ions through Fenton reaction (Lin and Wu, 2004). Generally, H_2O_2 did not cause any significant alteration of DNA molecules at physiological concentration. However, in the presence of transition metal ions, it is able to generate sufficient highly reactive oxygen species (viz. •OH and •OOH) which then cause severe damage to DNA, through induction of DNA strand breakage as well as base modification (Wood and Lee, 2005).

$$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + \bullet OH + OH^- \text{ (Oxidation)}$$

$$Fe^{3+} + H_2O_2 \text{ ----> } Fe^{2+} + \bullet OOH + H^+ \text{ (Reduction)}$$

Figure 4.22: The Fenton Reaction system (Brömme *et. al.*, 2002)

Figures 4.23 and 4.24 show the results of agarose gel electrophoresis of plasmid DNA incubated with 50 μM complex in TN buffer (pH 7.2) for 2 hours at 37°C in the presence of 30 μM hydrogen peroxide. Control experiments suggested that untreated DNA and DNA incubated with H_2O_2 alone did not show any significant DNA cleavage (Figure. 4.23, L2 – 3). Very little DNA cleavage (nicking of DNA) was observed for all the copper(II) complexes under this study without the addition of H_2O_2 in this experimental conditions (Figure. 4.23, L7 – 10; Figure 4.24, L4 – 5).

All six complexes, [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 possessed similar cleavage abilities as all of them were able to convert almost all the supercoiled DNA into the same amount of nicked form DNA, and the same small amount of linear form of DNA in the presence of H_2O_2 (Figure 4.23, L14 – 17; Figure 4.24, L7 – 8).

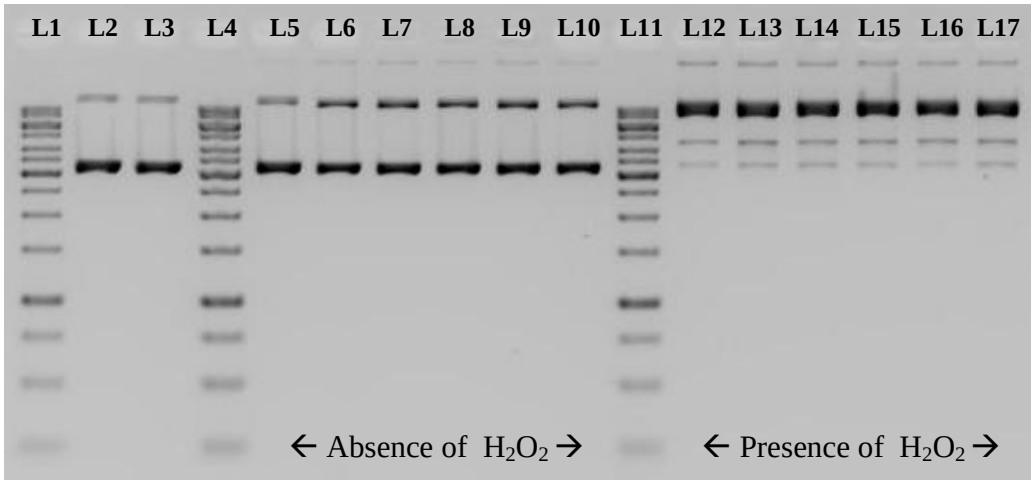


Figure 4.23: Electrophoresis results of separately incubating pBR322 (0.5 μg/μL) for 2 hours in TN buffer, pH 7.2 at 37°C with 50 μM copper(II) complexes in the presence and absence of H_2O_2. (L5, $CuCl_2$; L6, [Cu(phen)Cl_2]; L7, [Cu(phen)(D-ala)(H_2O)]Cl; L8, [Cu(phen)(L-ala)(H_2O)]Cl; L9, [Cu(phen)(D-threo)(H_2O)]Cl; L10, [Cu(phen)(L-threo)(H_2O)]Cl) and in the presence of 30 μM H_2O_2 (L12, $CuCl_2$; L13, [Cu(phen)Cl_2]; L14, [Cu(phen)(D-ala)(H_2O)]Cl; L15, [Cu(phen)(L-ala)(H_2O)]Cl; L16, [Cu(phen)(D-threo)(H_2O)]Cl; L17, [Cu(phen)(L-threo)(H_2O)]Cl). Lane 1, 4 & 11, Gene Ruler 1 Kb DNA Ladder; L2, Untreated DNA control; L3, DNA + 30 μM H_2O_2.

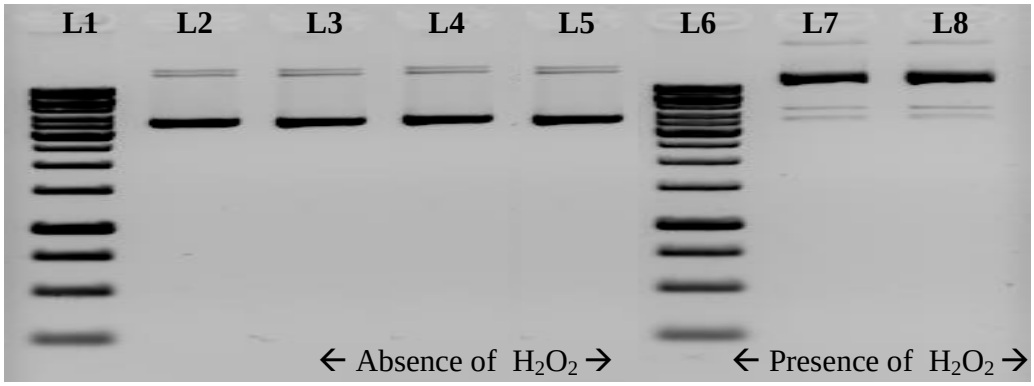


Figure 4.24: Electrophoresis results of separately incubating pBR322 (0.5 μg/μL) for 2 hours in TN buffer, pH 7.2 at 37°C with 50 μM copper(II) complexes in the presence and absence of H_2O_2. (L4, [Cu(phen)(D-threo)(H_2O)]NO_3; L5, [Cu(phen)(L-threo)(H_2O)]NO_3) and in the presence of 30 μM H_2O_2 (L7, [Cu(phen)(D-threo)(H_2O)]NO_3; L8, [Cu(phen)(L-threo)(H_2O)]NO_3). L1 & 6,

Gene Ruler 1 Kb DNA Ladder; L2, Untreated DNA control; L3, DNA + 30 μM H_2O_2.

Cejudo *et al.*, 2005, suggested that the free hydroxyl radicals were bound to DNA due to the reaction of reduced copper(II) complexes with H_2O_2, caused single and/ or double strands breakage in DNA. The supercoiled plasmid DNA, pUC18, was converted to nicked or linear DNA with the addition of H_2O_2. With a further increase in concentration of H_2O_2 from 0.075 to 0.10 mM, the nucleolytic efficiency of copper(II) complexes of *N*-substituted thiazole sulfonamides was found to increase.

Nuclease activity exhibited by copper(II) complexes in the presence of hydrogen peroxide has also been attributed to the participation of hydroxyl radical in DNA cleavage (Ng *et al.*, 2006; Uma *et al.*, 2005). As the H_2O_2 concentration increases, the amount of double-strand scissions was enhanced due to the formation of more and more free hydroxyl radicals during the reduction reaction between the copper(II) complexes and H_2O_2. Similar results were reported for DNA strand cleavage induced by other copper(II) complexes in the presence of H_2O_2 (Duarte and Jones, 2007).

This phenomenon can be explained by referring to Fenton-Weiss-type reactions. In these reactions, a transition metal ion reduces H_2O_2 to yield hydroxyl radical. The hydroxyl radicals produced can damage DNA and leads to strand breakage, depurination/ depyrimidation, and chemical modification of the bases or sugar, in addition to lipid peroxidation and protein modification (Duarte and

Jones, 2007). In addition, in the presence of H_2O_2, the hydoxo-bridged dinuclear copper(II)/ phen (1,10-phenanthroline) complex formed were found to exhibit nuclease activity and this also indicates that the formation of free radical during the oxidative reaction (Lü *et al.*, 2005).

Arranz *et al.*, 2007 also reported that the increasing concentration of H_2O_2 (10 – 100 μM for 5 minutes or 10 – 50 μM for 30 minutes) induced a significant increase of DNA strand breaks. Human hepatocellular carcinoma (HepG2) cells treated with a concentration of 10 μM H_2O_2 for 5 – 30 minutes markedly increased the DNA strand breaks in Formamidopyrimidine-DNA glycosylase (Fpg) sensitive sites, whereas the Endonuclease III (Endo III) sensitive sites were slightly increased. Maximum increase of DNA strand breaks at Fpg sensitive sites and Endo III sensitive sites occurred when the reaction mixture was incubated in the presence of 50 μM H_2O_2 for 30 minutes (Arranz *et al.*, 2007).

4.1.2.2 Nucleolytic study in the present of L-Ascorbic acid

Ascorbic acid (vitamin C) is an essential micronutrient and is considered as the most important water-soluble antioxidant in human plasma. The antioxidant property of ascorbic acid is often considered responsible for its effects against cardiovascular disease, certain types of cancers, and reduces tocopherol radicals back to their active form at the cellular membranes. Besides, as a reducing agent, it can directly scavenge superoxide radical, singlet oxygen, hydrogen peroxide and hydroxyl radical (Duarte and Jones, 2007; Klimczak *et. al.,* 2007).

Figures 4.25 and 4.26 showed the results of agarose gel electrophoresis of plasmid DNA induced by 50 μM copper(II) complexes, incubated in TN buffer (pH 7.2) for 2 hours at 37°C in the presence of 5 μM L-ascorbic acid (AA). Similarly, control experiments suggested that untreated DNA and DNA incubated with L-ascorbic acid alone did not show any significant DNA cleavage (Figure. 4.25, L2 – 3). No significant DNA cleavage is observed for all the copper(II) complexes in this study without the addition of AA in this experimental condition (Figure. 4.25 L7 – 10; Figure 4.26, L4 – 5). With the presence of 5 μM AA, the cleavage efficiency for all six complexes, which are [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3, had obviously increased. All supercoiled DNA has been converted into nicked and linear form of DNA (Figure 4.25, L14 – 17; Figure 4.26, L7 – 8).

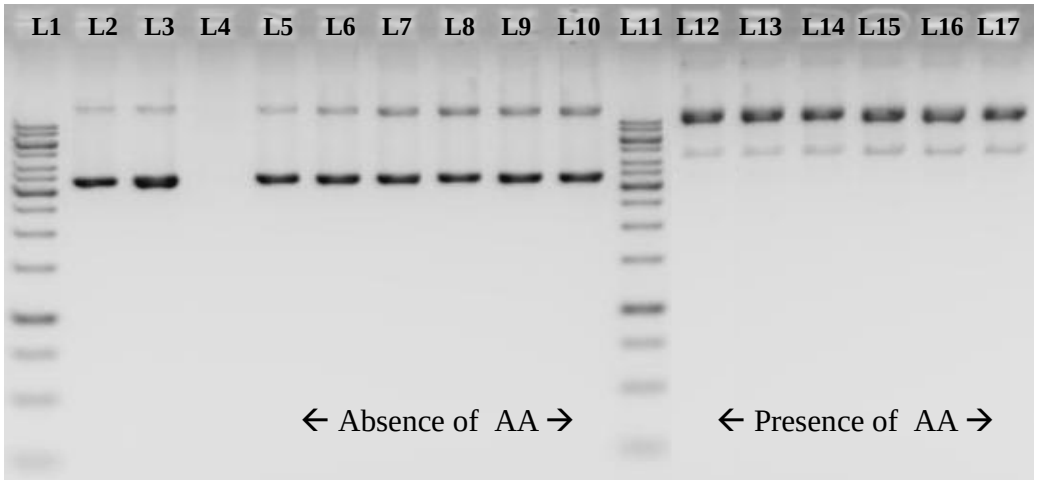


Figure 4.25: Electrophoresis results of separately incubating pBR322 (0.5 μg/μL) for 2 hours in TN buffer, pH 7.2 at 37^{o}C with 50 μM copper(II) complexes in the presence and absence of L-ascorbic acid (AA). (L5, $CuCl_2$; L6, [Cu(phen)Cl_2]; L7, [Cu(phen)(D-ala)(H_2O)]Cl; L8, [Cu(phen)(L-ala)(H_2O)]Cl; L9, [Cu(phen)(D-threo)(H_2O)]Cl; L10, [Cu(phen)(L-threo)(H_2O)]Cl) and in the presence of 2 μM AA (L12, $CuCl_2$; L13, [Cu(phen)Cl_2]; L14, [Cu(phen)(D-ala)(H_2O)]Cl; L15, [Cu(phen)(L-ala)(H_2O)]Cl; L16, [Cu(phen)(D-threo)(H_2O)]Cl; L17, [Cu(phen)(L-threo)(H_2O)]Cl). Lane 1 & 11, Gene Ruler 1 Kb DNA Ladder; L2, Untreated DNA control; L3, DNA + 2 μM AA; L4, Empty.

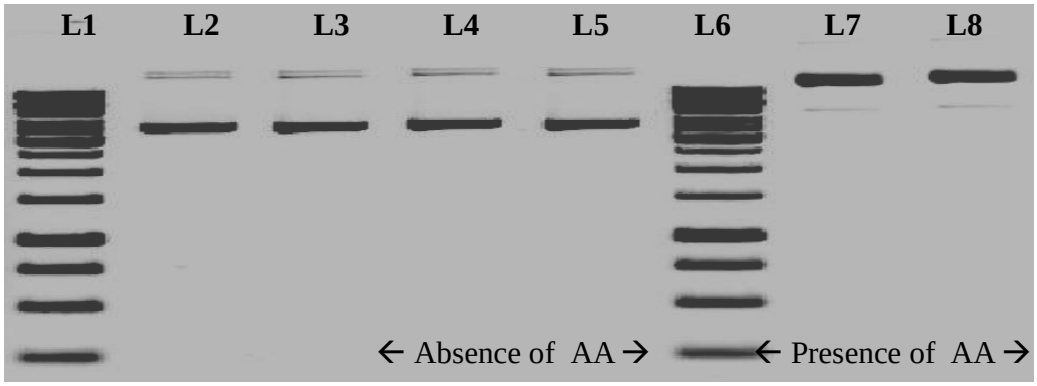


Figure 4.26: Electrophoresis results of separately incubating pBR322 (0.5 μg/μL) for 2 hours in TN buffer, pH 7.2 at 37^{o}C with 50 μM copper(II) complexes in the presence and absence of L-ascorbic acid (AA). (L4, [Cu(phen)(D-threo)(H_2O)]NO_3; L5, [Cu(phen)(L-threo)(H_2O)]NO_3) and in the presence of 2 μM AA (L7, [Cu(phen)(D-threo)(H_2O)]NO_3; L8, [Cu(phen)(L-threo)(H_2O)]NO_3). L1 & 6, Gene Ruler 1 Kb DNA Ladder; L2, Untreated DNA control; L3, DNA + 2 μM AA.

In 1999, Sreedhara *et al.* stated that DNA cleavage has been induced oxidatively by copper(II)/Neamine complex and copper(II)/Kanamycin A complex in the presence of H_2O_2 or ascorbic acid. Interestingly, copper(II)/Kanamycin A complex was also found to exhibit DNA cleavage in the absence of reducing agent, *via* a hydrolytic degradation pathway at the higher complex concentration used in those studies (Sreedhara *et al.*, 1999). Besides that, the mechanistic study into the mechanism of cytotoxicity of the anticancer copper(II) complex of 4,7-dimethyl-1,10-phenanthroline and glycinate shows strong binding of the complex to DNA and degrades DNA in the presence of reducing agents (Müller *et al.*, 2007).

Routier *et al.*, 1998, who studied the nucleolytic property of a series of metal complexes of 2,9-bis(2-hydroxyphenyl)-1,10-phenanthroline (M = Cu(II), Co(II), Ni(II) and Mn(III)), claimed that the cleavage of DNA by cobalt(II) complex in the presence of reducing agent such as ascorbic acid. Moreover, a novel ternary copper(II) complex, $[Cu(phen)(L\text{-threonine})(H_2O)](ClO_4)$, was shown to exhibit potent cytotoxic effects against various types of normal human as well as cancer cell line at a particular concentration. In the presence of ascorbate, pBR 322 DNA was found to be cleaved by the complex (Zhang *et al.*, 2004).

According to the DNA cleavage mechanism proposed by Sigman *et al.*, 1991, for $[Cu(phen)_2]^{2+}$ complex, a copper(II) complex was first reduced by

ascorbic acid to form the Cu(I) species, which then bound to DNA forming a Cu(I) complex-DNA adduct (Sigman *et al.*, 1991). The latter then reacted with hydrogen peroxide to form a 'copper-oxene' radical, which was the species responsible for the cleavage of DNA (Baron *et al.*, 1936). It was also possible that the copper(II) complex freely diffused to bind to DNA, the DNA-bound copper(II) complex approached the deoxyl ribose moiety in the minor groove and then was reduced by ascorbic acid to Cu(I) form. The DNA-bound Cu(I) form then reacted with H_2O_2 or other O_2-derived species generated in the presence of a reductant and dioxygen to form a hydroxyl radical, which then brings about the abstraction of the C-1 hydrogen of the deoxyl ribose moiety (Douglas, 1997).

Furthermore, Marcías *et al.*, 2005, have stated that transition metal ions serve as bridge for the reaction of ascorbic acid and oxygen through the d orbital of the metals. On the other hand, the radical form of ascorbic acid (ascorbyl radical) can react with oxygen to produce oxygen radicals. Hence, the concentration or the amount of complex poses a direct effect in the induction of ascorbyl radical which leads to DNA cleavage (Marcías *et al.*, 2005). Similar mechanism may be responsible for DNA cleavage by the copper complexes in this investigation.

Moreover, Seng *et al.*, 2008, found that in the absence of ascorbic acid, all the [M(phen)(edda)] (M = Cu, Co, Ni, Zn) complexes (50 μM) did not induce any DNA cleavage. However, addition of 1 μM ascorbic acid to the 50 μM

[Cu(phen)(edda)] greatly enhanced the DNA cleavage as nearly all the supercoiled plasmid was converted to nicked and linear DNA (Seng *et al.*, 2008). An acyclic copper(II) complex had been found to behave in a similar manner to [Cu(phen)(edda)]. This acyclic copper(II) complex alone, without added exogenous agent, could cleave DNA at sufficiently high micromolar concentration. At 25 μM, the acyclic copper(II) complex was inactive. When 100 μM of this complex was incubated with ascorbic acid, the DNA cleavage was enhanced in comparison with that by 100 μM complex alone (Xu *et al.*, 2004).

4.2 DNA binding study

The interaction of transition metal ions and complexes with DNA has been extensively studied many years ago (Hartwig *et al.*, 2002; Stubbe and Kozarich, 1987; Burrows and Rokita, 1994; Banerjee *et al.*, 1993; Pyle and Barton, 1990; Klein *et al.*, 1991; Kasprzak, 1991). Generally, metal ions and complexes have been identified to have a natural aptitude for interacting with DNA due to their cationic character, three-dimensional (3-D) structural profiles and the anionic nature of DNA (Detty *et al.*, 2004).

Various types of interaction of metal(II) complexes with DNA are known. Some important binding modes are (i) electrostatic interaction that involves the attraction between the cationic metal(II) complex with the negatively charged DNA phosphates backbone, (ii) interaction with grooves of DNA, and (iii) intercalation interaction which causes unwinding of the base pairs of DNA to accommodate the intercalating agent (Sigman, 1990; Ames *et al.*, 1993; Jiao *et al.*, 2005). Binding of these complexes to DNA can be stabilized through various kinds of interaction, such as π-stacking associated with the intercalation of aromatic heterocyclic groups between the base pairs, hydrogen bonding, van der Waals interactions of functionalities bound along the grooves of DNA helix, and hydrophobic effects (Kumar *et al.*, 2008).

The interaction of a set of chiral copper(II) complexes with DNA was extensively investigated in this section. Two main methods were used to study the

DNA binding properties of copper(II) complexes, i.e. fluorescence intercalator quenching (FIQ) assay and restriction enzyme inhibition assay. Ethidium bromide (EB) quenching assay was used to determine the apparent binding constant (K_{app}) of copper(II) complexes towards different kinds of DNA (Wang *et al*., 2005; Yuan *et al*., 2006; Li *et al*., 2007).

4.2.1 Comparative DNA binding study of [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 with calf thymus (CT) DNA

The comparative study involves six copper(II) complexes with different types of chiral ligands, viz [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen) (L-threo)(H_2O)]NO_3. The effect of the ligand (changing the subsidiary ligand) in this series of copper(II) complexes was investigated by EB quenching assay.

The binding strength of the copper(II) complexes with CT-DNA were determined quantitatively through EB quenching assay. The apparent binding constant, K_{app}, of each complex was calculated as explained in Section 3.5.1. The CT-DNA apparent binding constants of this series of copper(II) complexes are given in Table. 4.1).

Table 4.1 : Apparent binding constants of [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 on CT-DNA.

Complex	**Concentration at 50% quenching/ μM**	**Apparent binding constant (K_{app}) M^{-1}**
[Cu(phen)(D-ala)(H_2O)]Cl	38.76	$2.58 \pm 0.04 \times 10^5$
[Cu(phen)(L-ala)(H_2O)]Cl	31.43	$3.19 \pm 0.21 \times 10^5$
[Cu(phen)(D-threo)(H_2O)]Cl	48.49	$2.06 \pm 0.05 \times 10^5$
[Cu(phen)(L-threo)(H_2O)]Cl	54.38	$1.84 \pm 0.08 \times 10^5$
[Cu(phen)(D-threo)(H_2O)]NO_3	49.16	$2.03 \pm 0.02 \times 10^5$
[Cu(phen)(L-threo)(H_2O)]NO_3	56.49	$1.78 \pm 0.19 \times 10^5$

Competitive EB binding study was undertaken to determine quantitatively the binding affinity of the copper(II) complexes. The molecular fluorophore EB emits intense fluorescence in the presence of CT-DNA due to its protection from quenching by water or solvent molecules. In certain condition, addition of a second molecule would lead to displacement of EB from DNA and quenching of the EB emission (Yuan *et al*., 2006; Li *et al*., 2007). The reduction of the emission intensity of DNA-EB system on increasing the complex concentration can be due to displacement of the DNA bound EB by the copper(II) complexes, and this phenomenon is commonly known as quenching effect. The apparent binding constants of the copper(II) complexes were calculated from the classical Stern-

Volmer equation at 50% quenching of DNA-bound EB. From these values, the order of increasing binding strength of the copper(II) complexes was found to be [Cu(phen)(L-ala)(H_2O)]Cl (K_{app} = 3.19 ± 0.21 x 10^5 M^{-1}) > [Cu(phen)(D-ala)(H_2O)]Cl (K_{app} = 2.58 ± 0.01 x 10^5 M^{-1}) > [Cu(phen)(D-threo)(H_2O)]Cl (K_{app} = 2.06 ± 0.05 x 10^5 M^{-1}) > [Cu(phen)(D-threo)(H_2O)]NO_3 (K_{app} = 2.03 ± 0.02 x 10^5 M^{-1}) > [Cu(phen)(L-threo)(H_2O)]Cl (K_{app} = 1.84 ± 0.08 x 10^5 M^{-1}) > [Cu(phen)(L-threo)(H_2O)]NO_3 (K_{app} = 1.78 ± 0.19 x 10^5 M^{-1}), respectively (Table 4.1).

The complex [Cu(phen)(L-ala)(H_2O)]Cl binds more strongly compared to [Cu(phen)(D-ala)(H_2O)]Cl with the binding constant of 3.19 ± 0.21 x 10^5 M^{-1} and 2.58 ± 0.01 x 10^5 M^{-1}, respectively. This may due to the L-isomer is one of the 22 proteinogenic amino acids, i.e. building blocks of proteins (Doolittle, 1989). Comparing between subsidiary ligands, i.e. alanine and threonine, alanine bind more strongly compared to threonine. Moreover, the α-group of alanine and threonine are different. Alanine having a methyl group (hydrophobic) while threonine having a hydroxyl-alkyl group (hydrophilic), this differences of hydrophobicity may also affect the binding strength of copper(II) complexes.

In addition, the binding constant for [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3, as well as [Cu(phen)(L-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]NO_3 are found to be very similar and close to each other (Table 4.1). This phenomenon suggests that the anion, Cl^- and NO_3^-, does

not possess important effect on this DNA interaction study. These anions are probably dissociated from the cationic copper(II) complex in aqueous solution and exist as free ions.

From this study, it showed that the metal complexes with different subsidiary ligands can induce a change in the binding affinity.

4.2.2 DNA sequence selectivity and restriction enzyme inhibition studies

Controlling gene expression with small DNA-binding molecules has been a challenge at the interface of medicinal chemistry and biology. To achieve this goal, a number of chemical approaches have been used to search for small molecules that can selectively bind to DNA and either activate or inhibit gene expression (Qin *et al*., 2006). Efforts have focused on the rational design of ligands capable of binding tightly and specifically to any base sequence of double stranded DNA. These agents can potentially have wide application in elucidating the mechanism of action of antitumor and antivirus drugs, and developing chemotherapeutic agents (Oleksi *et al*., 2006; Lavalley *et al*., 2007; Pascu *et al*., 2008).

Over the past ten years, attention has been focused on targeting the AT-rich sequences of DNA. Some small molecules have been reported to be used extensively for biomedical applications, such as antiviral antibiotics. They exert significant biological activity by interfering with the proteins that regulate DNA

replication and transcription processes by their selective binding to the AT-rich sequences of DNA (Patra *et al*., 2007). In addition, natural ligands, like neotropsin and distamycin A, are found to have remarkable affinity to the DNA minor groove with AT-rich sequences (Boger *et al*., 2000; Tkadlecová *et al*., 2008).

In recent years, much interest has focused on the specific DNA sequence binding of mixed-ligand complexes which contain phen and the modified amino acid ligands which are to design to achieve more effective binding affinity of the complexes to specific DNA sequence (Mudasir *et al*., 2003). Further detailed studies using various central metal ions are needed to explore the influence of geometry, charge, spin state, redox potential, etc., on the DNA binding of the mixed-ligand complexes.

The aim of this study is to investigate the binding affinity and DNA sequence selectivity of a series of copper(II) complexes with different coordinated/ subsidiary chiral ligand(s). The DNA apparent binding constants of all the copper(II) complexes were calculated for DNA duplexes with different sequences such as $(CG)_6$ and $(AT)_6$, through EB quenching assay. In addition, restriction enzyme inhibition assay was carried out to investigate whether the copper(II) complexes binds randomly or selectively to specific sites or regions of the DNA.

4.2.2.1 Comparative DNA binding study of [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 with oligonucleotides ds$(AT)_6$ and ds$(CG)_6$

The study of binding and DNA sequence selectivity of copper(II) complexes, [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3, were carried out. The function and effect of the chiral subsidiary ligand and counter ion in copper(II) complexes were investigated by EB quenching assay to study the binding selectivity of copper(II) complexes on DNA.

The first part of this investigation was to find out the DNA binding preference for CG or AT rich sequences by complexes [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen) (L-threo)(H_2O)]NO_3. The K_{AT}/K_{CG} ratio is calculated by dividing the apparent binding constant of copper(II) complex on ds$(AT)_6$ DNA over the apparent constant on ds$(CG)_6$ DNA. The K_{AT}/K_{CG} ratio indicates the binding selectivity of copper(II) complexes on ds$(AT)_6$ DNA sequence over the ds$(CG)_6$ DNA sequence. The lower the K_{AT}/K_{CG} ratio value indicates low selectivity towards ds$(AT)_6$ DNA sequence.

From the experimental result of EB quenching assay on $ds(CG)_6$ and $ds(AT)_6$ oligonucleotides (Table 4.2), complexes [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen) (L-threo)(H_2O)]NO_3 showed significantly higher apparent binding constants (i.e., $5.18 \pm 0.06 \times 10^4$ M^{-1}, $8.26 \pm 0.16 \times 10^4$ M^{-1}, $2.81 \pm 0.11 \times 10^4$ M^{-1}, and $2.47 \pm 0.04 \times 10^4$ M^{-1} respectively) for $ds(AT)_6$ than $ds(CG)_6$, this suggested that they binds preferentially or more selectively to $ds(AT)_6$.

On the other hand, complexes [Cu(phen)(D-threo)(H_2O)]Cl, and [Cu(phen)(L-threo)(H_2O)]Cl show no preference to either $ds(AT)_6$ or $ds(CG)_6$ oligonucleotides as the binding constants are very close to each other (i.e., $3.24 \pm 0.06 \times 10^4$ M^{-1} and $3.20 \pm 0.02 \times 10^4$ M^{-1} ; $2.45 \pm 0.10 \times 10^4$ M^{-1}, and $3.05 \pm 0.05 \times 10^4$ M^{-1}, respectively).

Table 4.2: EB quenching assay results of [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 on ds$(AT)_6$ and ds$(CG)_6$ oligonucleotides.

Complex	**Apparent binding constant (K_{app}) M^{-1} on ds$(AT)_6$**	**Apparent binding constant (K_{app}) M^{-1} on ds$(CG)_6$**	**K_{AT}/K_{CG}**
[Cu(phen)(D-ala)(H_2O)]Cl	$5.18 \pm 0.06 \times 10^4$	$3.35 \pm 0.11 \times 10^4$	1.55
[Cu(phen)(L-ala)(H_2O)]Cl	$8.26 \pm 0.16 \times 10^4$	$2.95 \pm 0.04 \times 10^4$	2.80
[Cu(phen)(D-threo)(H_2O)]Cl	$3.24 \pm 0.06 \times 10^4$	$3.20 \pm 0.02 \times 10^4$	1.01
[Cu(phen)(L-threo)(H_2O)]Cl	$2.45 \pm 0.10 \times 10^4$	$3.05 \pm 0.05 \times 10^4$	0.80
[Cu(phen)(D-threo)(H_2O)]NO_3	$2.81 \pm 0.11 \times 10^4$	$1.50 \pm 0.01 \times 10^4$	1.87
[Cu(phen)(L-threo)(H_2O)]NO_3	$2.47 \pm 0.04 \times 10^4$	$1.55 \pm 0.02 \times 10^4$	1.59

- **K_{AT}/K_{CG} = ratio of K_{app}, ds$(AT)_6$/ K_{app}, ds$(CG)_6$**

The complex [Cu(phen)(L-ala)(H_2O)]Cl binds strongest on ds$(AT)_6$ oligonucleotides and possessed strongest apparent binding constant ($8.26 \pm 0.16 \times 10^4$ M^{-1}) among the six copper(II) complexes. Between the set of alanine-containing complexes, [Cu(phen)(L-ala)(H_2O)]Cl showed stronger binding affinity towards ds$(AT)_6$ oligonucleotides than its enantiomeric complex, [Cu(phen)(D-ala)(H_2O)]Cl. The K_{AT}/K_{CG} of [Cu(phen)(L-ala)(H_2O)]Cl (2.80) is about two folds greater than complex [Cu(phen)(D-ala)(H_2O)]Cl (1.55). This may

due to the chirality of the copper(II) complexes and the coordination of L-isomer. In pharmacology, chirality is an important factor in determine binding strength and drug efficacy (Montana *et al*., 2008). As all the naturally occurring amino acids are L-isomers, the presence of L-alanine in [Cu(phen)(L-ala)(H_2O)]Cl may make it bind to DNA more strongly.

In addition, the anion, Cl^- and NO_3^-, are proposed to dissociate from the cationic copper(II) complex in aqueous solution and exist as free ions. However, these anions are found to be affect the DNA binding study. In this case, the copper(II) complexes with Cl^-, e.g. [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl are found to bind stronger to DNA compared to the copper(II) complexes with NO_3^-, e.g. [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3. It is suggested that the size and molecular weight of Cl^- is much more smaller than NO_3^-, the steric hindrance and screening effect of Cl^- is lower, and hence, the copper(II) complexes with Cl^- bind stronger to DNA.

According to the ratio of K_{app}, ds$(AT)_6$ / K_{app}, ds$(CG)_6$, ie. K_{AT}/K_{CG}, of this series of copper(II) complexes in Table 4.2, the order of selectivity towards ds$(AT)_6$ is [Cu(phen)(L-ala)(H_2O)]Cl > [Cu(phen)(D-threo)(H_2O)]NO_3 > [Cu(phen)(L-threo)(H_2O)]NO_3 > [Cu(phen)(D-ala)(H_2O)]Cl > [Cu(phen)(D-threo)(H_2O)]Cl > [Cu(phen)(L-threo)(H_2O)]Cl (K_{AT}/K_{CG} = 2.80 > 1.87 > 1.59 > 1.55 > 1.01 > 0.80, respectively) (Table 4.2)

4.2.2.2 Restriction enzyme inhibition study of [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 complexes

Restriction endonuclease, or commonly known as restriction enzyme, is a type of enzyme that is able to cleave double stranded or single stranded DNA. The cleavage of DNA will take place at one or more than one, specific and unique recognized nucleotide sequences, which are known as restriction sites (Roberts *et al*., 1976).

1.Tsp 509I	5’—↓ A A T T —3’ 3’— T T A A ↑—5’	7. Ase I	5’—A T ↓T A A T—3’ 3’— T A A T ↑T A—5’
2. Hae III	5’—G C ↓G C —3’ 3’—C G ↑C G —5’	8. Ssp I	5’—A A ↓T A T T—3’ 3’— T T A T ↑A A—5’
3. Sal I	5’—G ↓T C G A C—3’ 3’—C A G C T ↑G—5’	9. Mun I	5’—C↓ A A T T G—3’ 3’— G T T A A ↑C—5’
4. Pst I	5’—C T G C A ↓G—3’ 3’—G↑ A C G T C—5’	10. EcoR I	5’—G↓ A A T T C—3’ 3’— C T T A A ↑G—5’
5. Pvu II	5’—C A G ↓C T G—3’ 3’—G T C ↑ G A C—5’	11. NdeI	5’—C A↓ T A T G—3’ 3’—G T A T ↑A C—5’
6. Sca I	5’—A G T ↓A C T—3’ 3’—T C A ↑ T G A—5’	12. Bst 11071	5’—G T A ↓ T A C—3’ 3’—C A T ↑ A T G—5’

All the above twelve restriction enzymes were used to examine the binding selectivity and specificity (i.e., recognize specific nucleotide sequences) of six copper(II) complexes.. Basically, if the bases at the restriction site(s) are bonded or masked by the metal complex, the restriction enzyme cannot recognize

the nucleotide sequence and hence is unable to cleave the λ DNA anymore (Karidi *et al*., 2002; Karidi *et al*., 2005). In this experiment, we can briefly conclude that whether there is inhibition of DNA cleavage by restriction enzyme or alteration of DNA cleavage.

Control experiment suggests that $CuCl_2$ salt did not alter the restriction enzyme activity as no prevention of restriction enzyme digestion was observed (Figure 4.27 – 4.28). This may due to $CuCl_2$ been unable to recognize, bond or mask the specific nucleotide sequences in the λ DNA, and thus did not prevent DNA digestion by restriction enzyme.

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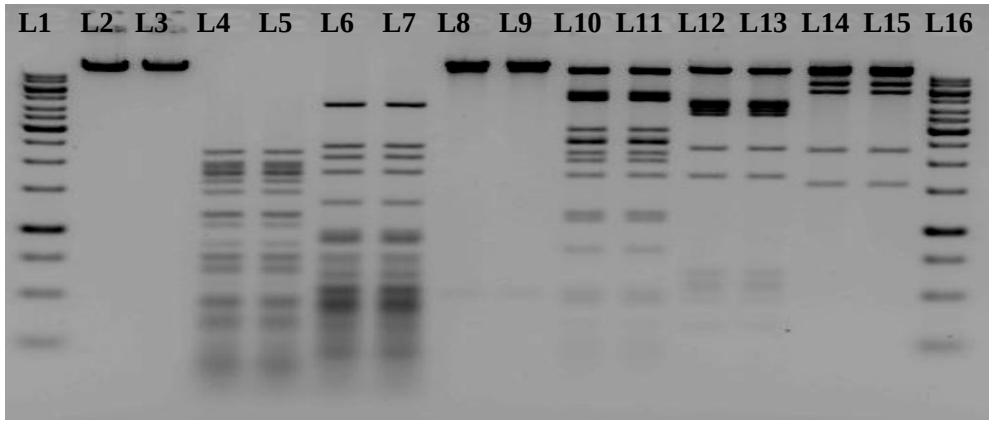

Figure 4.27: Electrophoresis results of incubating λ DNA (0.5 µg/µL) with 5 unit of restriction enzyme in the presence or absence of 50 µM $CuCl_2$ for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 µg); Lane 3, λ DNA + 50 µM metal salt; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 µM metal salt; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 µM metal salt; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 µM metal salt; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 µM metal salt; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 µM metal salt; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 µM metal salt; Lane 16, 1kb DNA ladder.

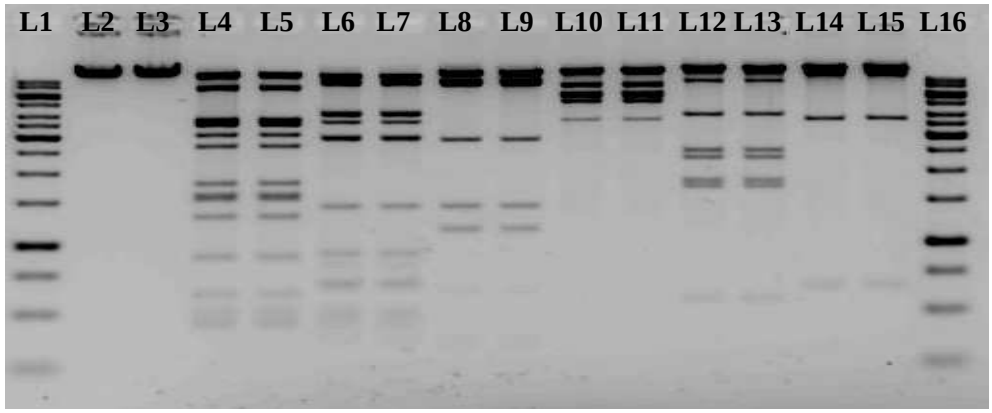

Figure 4.28: Electrophoresis results of incubating λ DNA (0.5 µg/µL) with 5 unit of restriction enzyme in the presence or absence of 50 µM $CuCl_2$ for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 µg); Lane 3, λ DNA + 50 µM metal salt; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 µM metal salt; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 µM metal salt; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 µM metal salt; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 µM metal salt; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 µM metal salt; Lane 14, λ DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 µM metal salt; Lane 16, 1kb DNA ladder.

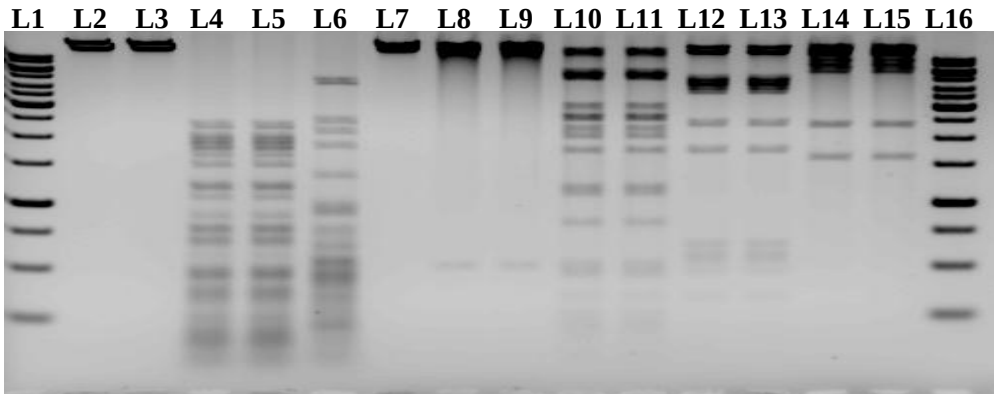

Figure 4.29: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)Cl_2] for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 μM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 μM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 μM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 μM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 μM metal complex; Lane 16, 1kb DNA ladder.

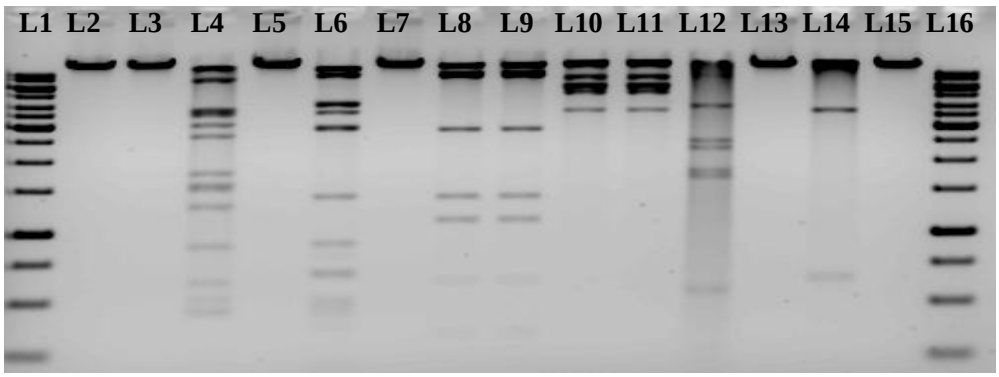

Figure 4.30: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)Cl_2] for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 μM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 μM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 μM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 μM metal complex; Lane 14, λ DNA + 5 unit Bst 11071 (control);

Lane 15, λ DNA + 5 unit Bst 11071 + 50 μM metal complex; Lane 16, 1kb DNA ladder.

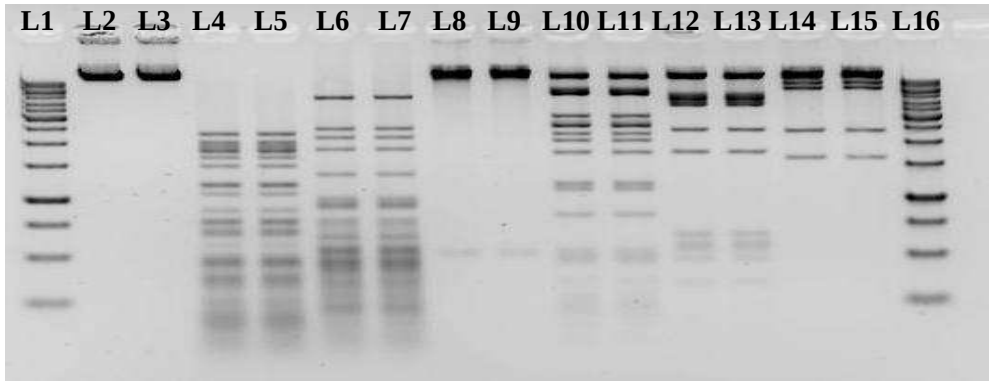

Figure 4.31: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(D-ala)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 μM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 μM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 μM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 μM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 μM metal complex; Lane 16, 1kb DNA ladder.

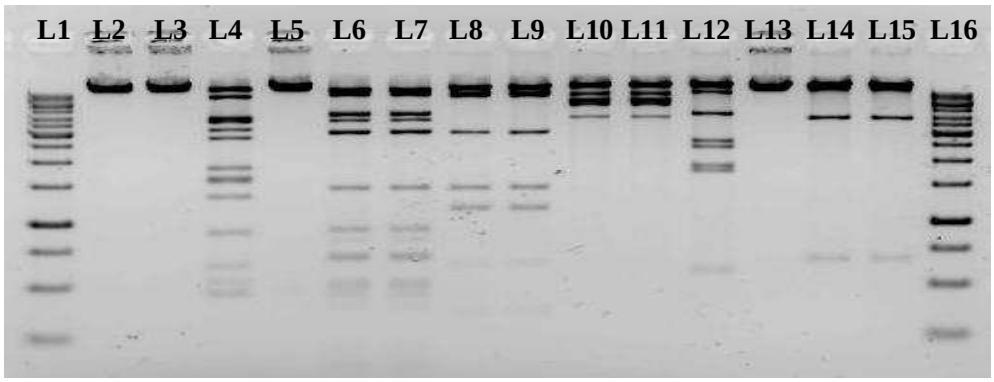

Figure 4.32: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(D-ala)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 μM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 μM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 μM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 μM metal complex; Lane 14, λ DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 μM metal complex; Lane 16, 1kb DNA ladder.

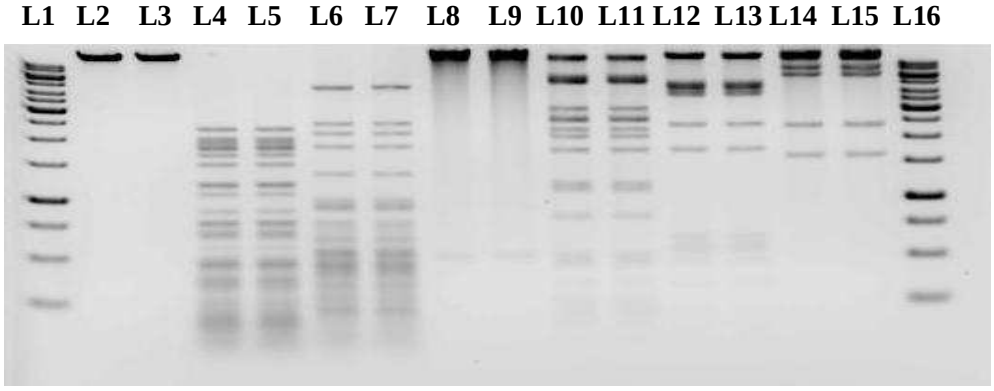

Figure 4.33: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(L-ala)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 μM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 μM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 μM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 μM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 μM metal complex; Lane 16, 1kb DNA ladder.

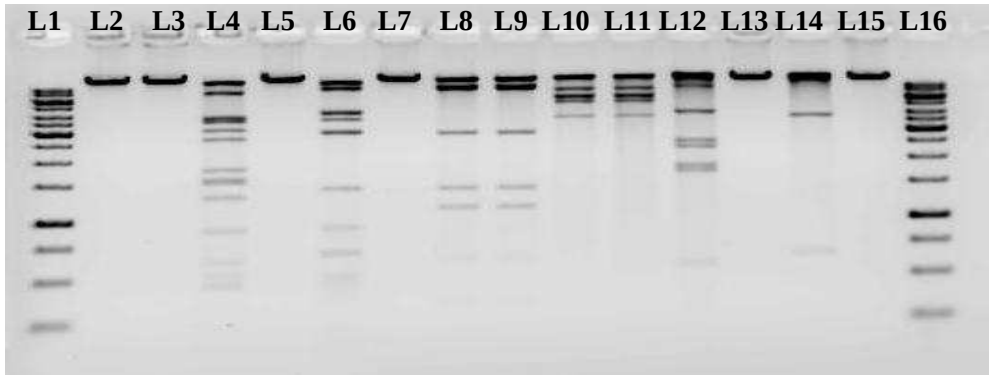

Figure 4.34: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(L-ala)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 μM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 μM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 μM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 μM metal complex; Lane 14, λ DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 μM metal complex; Lane 16, 1kb DNA ladder.

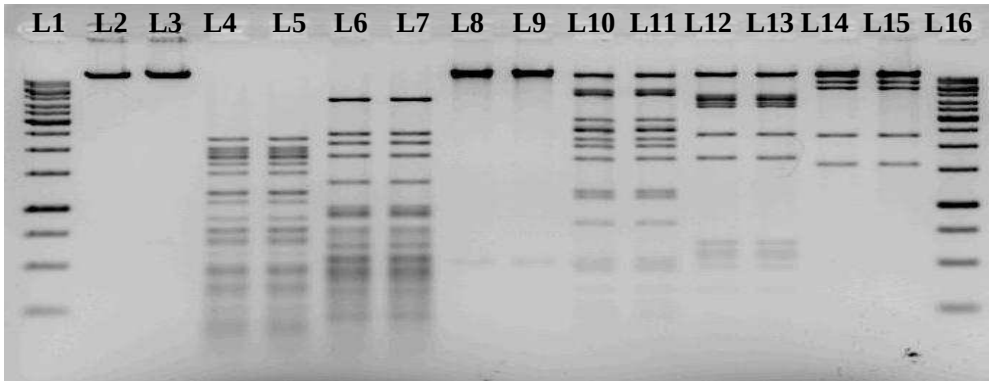

Figure 4.35: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(D-threo)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 μM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 μM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 μM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 μM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 μM metal complex; Lane 16, 1kb DNA ladder.

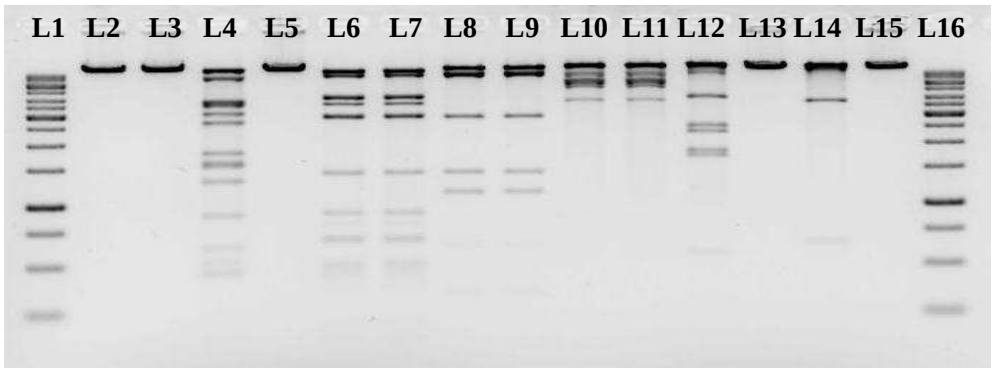

Figure 4.36: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(D-threo)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 μM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 μM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 μM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 μM metal complex; Lane 14, λ DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 μM metal complex; Lane 16, 1kb DNA ladder.

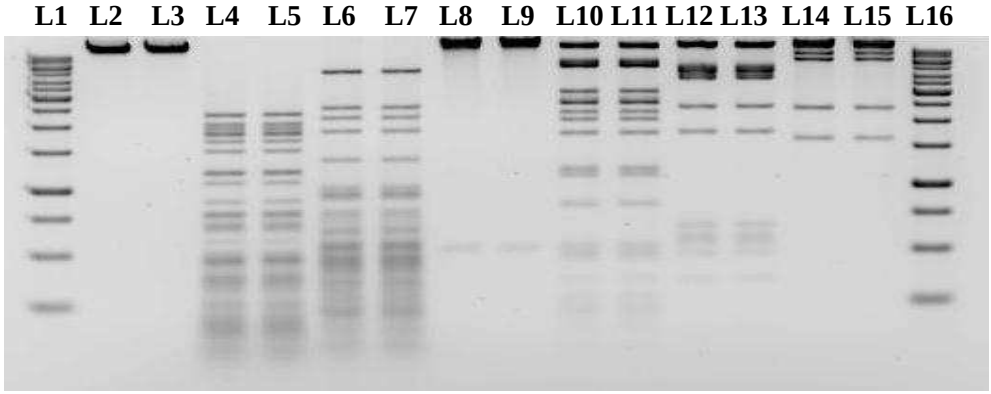

Figure 4.37: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(L-threo)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 μM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 μM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 μM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 μM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 μM metal complex; Lane 16, 1kb DNA ladder.

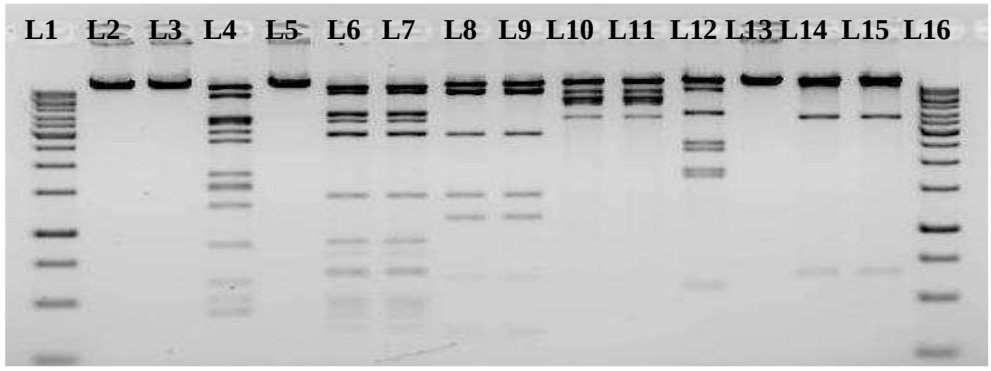

Figure 4.38: Electrophoresis results of incubating λ DNA (0.5 μg/μL) with 5 unit of restriction enzyme in the presence or absence of 50 μM [Cu(phen)(L-threo)(H_2O)]Cl for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 μg); Lane 3, λ DNA + 50 μM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 μM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 μM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 μM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 μM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 μM metal complex; Lane 14, λ

DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 µM metal complex; Lane 16, 1kb DNA ladder.

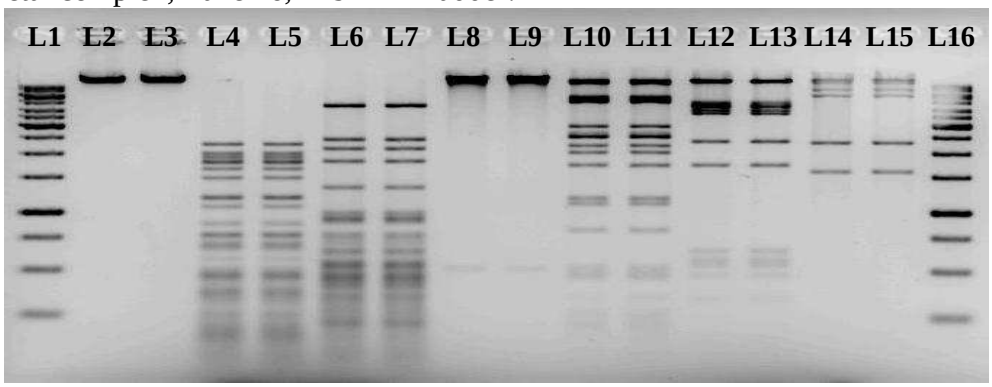

Figure 4.39: Electrophoresis results of incubating λ DNA (0.5 µg/µL) with 5 unit of restriction enzyme in the presence or absence of 50 µM [Cu(phen)(D-threo)(H_2O)]NO_3 for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 µg); Lane 3, λ DNA + 50 µM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 µM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 µM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 µM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 µM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 µM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 µM metal complex; Lane 16, 1kb DNA ladder.

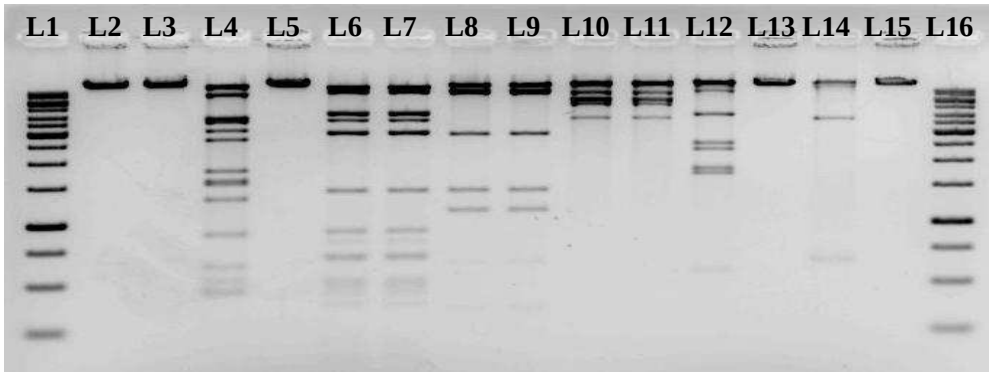

Figure 4.40: Electrophoresis results of incubating λ DNA (0.5 µg/µL) with 5 unit of restriction enzyme in the presence or absence of 50 µM [Cu(phen)(D-threo)(H_2O)]NO_3 for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 µg); Lane 3, λ DNA + 50 µM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 µM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 µM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 µM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 µM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 µM metal complex; Lane 14, λ

DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 μM metal complex; Lane 16, 1kb DNA ladder.

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 L11 L12 L13 L14 L15 L16

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 L11 L12 L13 L14 L

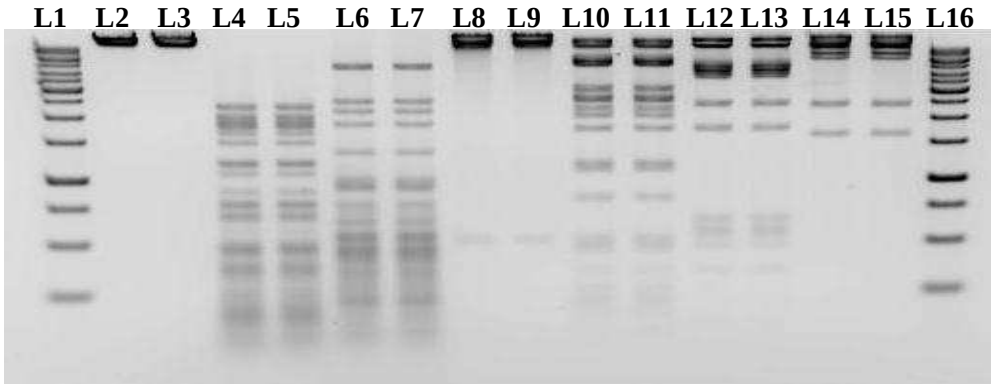

Figure 4.41: Electrophoresis results of incubating λ DNA (0.5 µg/µL) with 5 unit of restriction enzyme in the presence or absence of 50 µM [Cu(phen)(L-threo)(H_2O)]NO_3 for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 µg); Lane 3, λ DNA + 50 µM metal complex; Lane 4, λ DNA + 5 unit Tsp 509I (control); Lane 5, λ DNA + 5 unit Tsp 509I + 50 µM metal complex; Lane 6, λ DNA + 5 unit Hae III (control); Lane 7, λ DNA + 5 unit Hae III + 50 µM metal complex; Lane 8, λ DNA + 5 unit Sal I (control); Lane 9, λ DNA + 5 unit Sal I + 50 µM metal complex; Lane 10, λ DNA + 5 unit Pst I (control); Lane 11, λ DNA + 5 unit Pst I + 50 µM metal complex; Lane 12, λ DNA + 5 unit Pvu II (control); Lane 13, λ DNA + 5 unit Pvu II + 50 µM metal complex; Lane 14, λ DNA + 5 unit Sca I (control); Lane 15, λ DNA + 5 unit Sca I + 50 µM metal complex; Lane 16, 1kb DNA ladder.

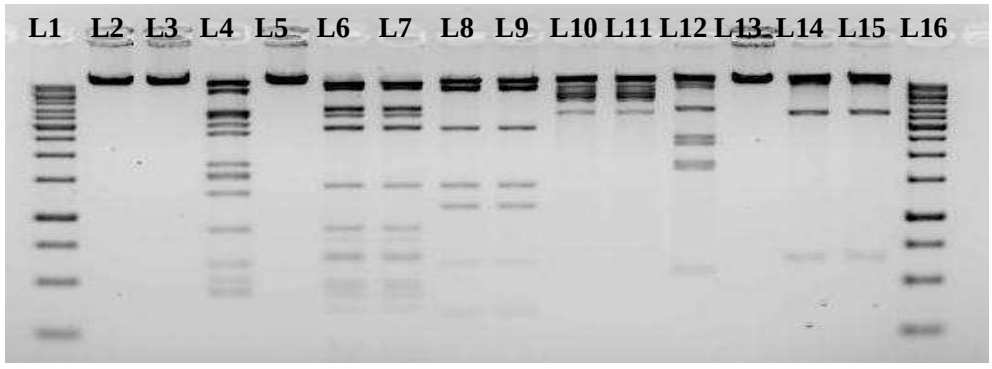

Figure 4.42: Electrophoresis results of incubating λ DNA (0.5 µg/µL) with 5 unit of restriction enzyme in the presence or absence of 50 µM [Cu(phen)(L-threo)(H_2O)]NO_3 for 2 hours at 37ºC. Lane 1, 1kb DNA ladder; Lane 2, λ DNA alone (0.5 µg); Lane 3, λ DNA + 50 µM metal complex; Lane 4, λ DNA + 5 unit Ssp I (control); Lane 5, λ DNA + 5 unit Ssp I + 50 µM metal complex; Lane 6, λ DNA + 5 unit Ase I (control); Lane 7, λ DNA + 5 unit Ase I + 50 µM metal complex; Lane 8, λ DNA + 5 unit Mun I (control); Lane 9, λ DNA + 5 unit Mun I + 50 µM metal complex; Lane 10, λ DNA + 5 unit EcoR I (control); Lane 11, λ DNA + 5 unit EcoR I + 50 µM metal complex; Lane 12, λ DNA + 5 unit NdeI (control); Lane 13, λ DNA + 5 unit NdeI + 50 µM metal complex; Lane 14, λ

DNA + 5 unit Bst 11071 (control); Lane 15, λ DNA + 5 unit Bst 11071 + 50 μM metal complex; Lane 16, 1kb DNA ladder.

Figure 4.29 – 4.30 showed the electrophoresis results of incubation λ DNA with 5 units of restriction enzyme in the presence or absence of 50 μM [Cu(phen)Cl_2] for 2 hours at 37ºC. The restriction enzymes Hae III, Ase I, Ssp I, Nde I and Bst 11071 which recognize the base sequences 5'-GCGC-3', 5'-ATTAAT-3', 5'-AATATT-3', 5'-CATATG-3' and 5'-GTATAC-3' respectively, were unable to digest the λ DNA into expected fragments in the presence of [Cu(phen)Cl_2] complex. In other words, the result indicated that complex [Cu(phen)Cl_2] was able to prevent these enzymes from cutting the λ DNA at these sequences 5'-GCGC-3', 5'-ATTAAT-3', 5'-AATATT-3', 5'-CATATG-3' and 5'-GTATAC-3'.

The result above suggested that the complex might bind to DNA bases and masked the unique recognition sites of these five restriction enzymes, and hence, these restriction enzymes were unable to recognize their DNA sequences anymore and digest the λ DNA into expected fragments (Karidi *et al*., 2002; Karidi *et al*., 2005; He *et al*., 2009).

Table 4.3: Inhibition of restriction enzymes activity by Copper(II) complexes, [Cu(phen)(aa)(H_2O)]X.

	Hae III	Ssp I	Ase I	Nde I	Bst 11071
$CuCl_2$ (Control)					
[Cu(phen)Cl_2] (Control)	√	√	√	√	√
[Cu(phen)(D-ala)(H_2O)]Cl		√		√	
[Cu(phen)(L-ala)(H_2O)]Cl		√	√	√	√
[Cu(phen)(D-threo)(H_2O)]Cl		√		√	√
[Cu(phen)(L-threo)(H_2O)]Cl		√		√	
[Cu(phen)(D-threo)(H_2O)]NO_3		√		√	√
[Cu(phen)(L-threo)(H_2O)]NO_3		√		√	

On the other hand, complex [Cu(phen)(L-ala)(H_2O)]Cl was able to inhibit the restriction enzyme activity of Ase I, Ssp I, Nde I, and Bst 11071. These four enzymes recognize base sequences 5'-ATTAAT-3', 5'-AATATT-3', 5'-CATATG-3' and 5'-GTATAC-3', respectively (Figure 4.37 – 4.38). Interestingly, the selectivity and specificity is improved when the L-ala ligand is replaced with its enantiomer, D-ala. The complex [Cu(phen)(D-ala)(H_2O)]Cl could inhibit the activity of only two restriction enzymes, which are Ssp I and Nde I, while [Cu(phen)(L-ala)(H_2O)]Cl could inhibit four. It seems that the chirality of coordinated amino acid can affect the ability of copper(II) complexes to inhibit particular type of restriction enzymes. Some research groups reported that chirality is an important factor in drug efficacy. About 56% drugs currently in use are chiral compounds, and about 88% of these chiral synthetic drug are used therapeutically as racemic (Rentsch, 2002; John, 2003). In addition, Montana *et al*.

(2002) also reported that the platinum(II) complex, cis-[(1S,2R,3S)-1,7,7-trimethylbicyclo[2.2.1]heptane-2,3-diamine]dichloroplatinum(II) was shown to bind stronger towards calf thymus (CT) DNA than its enantiomer, cis-[(1R,2S,3R)-1,7,7-trimethylbicyclo[2.2.1]heptane-2,3-diamine]dichloroplatinum (II).

The complex [Cu(phen)(D-threo)(H_2O)]Cl was able to inhibit the enzyme activity of Ssp I, Nde I, and Bst 11071 enzymes which recognize the base sequences 5'-AATATT-3', 5'-CATATG-3', and 5'-GTATAC-3', respectively (Figure 4.35 – 4.36). The complex with D-threo ligand was able to inhibit three restriction enzymes activity, while complex with L-threo ligand was able to inhibit only two enzymes activity, which are Ssp I and Nde I. The selectivity and specificity of complex is found to be slightly improved, when the D-threo ligand is replaced by its enantiomer, L-threo. Again, this suggests that the chirality of coordinated amino acid, will affect the binding selectivity and specificity of the copper(II) complex.

Furthermore, the results of complexes [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 are found to be exactly the same as the complexes [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl (Figure 4.35 – 4.42). Both [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3 were able to inhibit the enzyme activity of Ssp I, Nde I and Bst 11071 which recognize the base sequences 5'-AATATT-3', 5'-CATATG-3', and 5'-GTATAC-3',

respectively. On the other hand, both [Cu(phen)(L-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]NO_3 were able to inhibit activity of only two enzymes, which are Ssp I and Nde I that recognize the base sequences 5'-AATATT-3' and 5'-CATATG-3', respectively. This result can be explained that both Cl^- and NO_3^- anion might dissociated from the cationic copper(II) complex in aqueous solution and exist as free ions, the $[Cu(phen)(aa)(H_2O)]^+$ cations are the same and hence they doesn't show any effect in complex binding selectivity.

Lastly, we suggest that observed DNA recognition is conferred by the ability of the complexes to intercalate between the DNA bases and/or form hydrogen bonds with DNA through various hydrogen donor and acceptor groups on the subsidiary ligand. The chirality of subsidiary ligand(s), for example, replacement of D-amino acid with L- amino acid in copper(II) complexes may affect their ability in recognition and binding towards DNA. In short, the phenanthroline (phen) has recognition ability and the chirality of amino acid affects DNA binding selectivity to some extent.

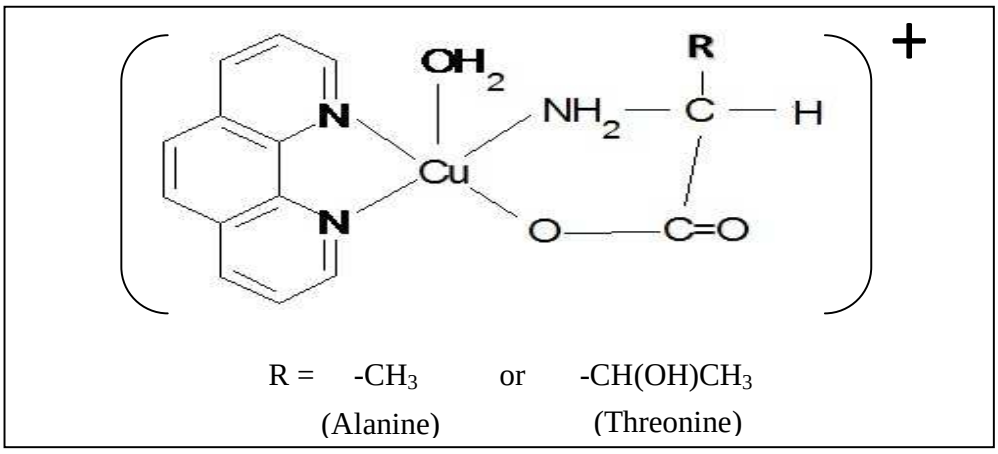

Figure 4.43: General structure of complex $[Cu(phen)(aa)(H_2O)]^+$.

4.3 G-quadruplex (telo21) Interaction Studies

G-quadruplexes are higher-order DNA and RNA structures formed from G-rich sequences that are built around tetrads of hydrogen-bonded guanine bases. They are further stabilized by the existence of a monovalent cation (especially potassium) in the center of the tetrads. Depending on the direction of the strands or parts of a strand that form the tetrads, structures may be described as parallel or antiparallel (Neidle & Balasubramanian, 2006). G-quadruplexes have become valid targets for new anticancer drugs in past few decades. Recent studies have demonstrated that small molecules can facilitate the formation of, and stabilize, G-quadruplexes. In addition, many leading compounds that target these structures have been reported, and a few of them have entered preclinical or clinical trials (Ou *et al.*, 2008; Han *et al.*, 2000).

Thiazole orange (TO) quenching assay was carried out to investigate the G-quadruplex DNA binding selectivity of copper(II) complexes, [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3. This assay has been extensively used to measure binding of small molecules to DNA (Wang *et al*., 2005; Yuan *et al*., 2006; Li *et al*., 2007). In this assay, the binding of the test compounds is monitored by a change in fluorescence due to the displacement of a bound fluorescent dye. Recently, it was shown that thiazole orange is an excellent dye for these studies since it is safer

than EB and binds equally well to both quadruplex and double stranded DNA (Monchaud *et al*., 2006; Paritala *et al*., 2009).

Thiazole orange quenching studies were conducted using oligo 22G 5'-$AG_3(T_2AG_3)_3$-3', and two complementary 17 nucleotide primers, 5'-CCAGTTCGTAGTAACCC-3' and 3'-GGTCAAGCATCATTGGG-5' (Monchaud *et al*., 2006; Paritala *et al*., 2009; Seng *et al*., 2009). The details of this method are explained in section 3.5.2. Binding constants and the ratio of quadruplex to duplex is shown in table 4.4.

Table 4.4 : Thiazole orange quenching assay studies to examine quadruplex binding by [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 ternary copper(II) complexes.

Complex	**Quadruplex DNA apparent binding constant 22G (M^{-1})**	**Duplex DNA apparent binding constant 17bp (M^{-1})**	**Ratio 22G/ 17bp**
[Cu(phen)(D-ala)(H_2O)]Cl	$9.33 \pm 0.03 \times 10^4$	$6.33 \pm 0.05 \times 10^4$	1.47
[Cu(phen)(L-ala)(H_2O)]Cl	$9.53 \pm 0.08 \times 10^4$	$6.53 \pm 0.03 \times 10^4$	1.46
[Cu(phen)(D-threo)(H_2O)]Cl	$7.49 \pm 0.05 \times 10^4$	$6.33 \pm 0.03 \times 10^4$	1.18
[Cu(phen)(L-threo)(H_2O)]Cl	$9.56 \pm 0.07 \times 10^4$	$6.41 \pm 0.06 \times 10^4$	1.49
[Cu(phen)(D-threo)(H_2O)]NO_3	$7.40 \pm 0.17 \times 10^4$	$5.89 \pm 0.06 \times 10^4$	1.24
[Cu(phen)(L-threo)(H_2O)]NO_3	$9.26 \pm 0.02 \times 10^4$	$7.42 \pm 0.08 \times 10^4$	1.25

In this study, the effect of varying the subsidiary aa-ligand in [Cu(phen)(aa)(H_2O)]X complexes (aa = amino acid, D-ala, L-ala, D-threo, L-threo; X = anion, Cl, NO_3) was investigated by thiazole orange quenching assay to assess the G-quadruplex selectivity of the [Cu(phen)(aa)(H_2O)]X complexes. The complex [Cu(phen)(L-threo)(H_2O)]Cl has the greatest binding affinity and selectivity towards G-quadruplex DNA with the highest 22G/17bp ratio of 1.49 and apparent binding constant on G-quadruplex DNA, K_{app}= 9.56 ± 0.07 x 10^4 (Table 4.4). The G-quadruplex DNA selectivity of complex [Cu(phen)(L-ala)(H_2O)]Cl is just a little bit lower than [Cu(phen)(L-threo)(H_2O)]Cl with the 22 G/17 bp ratio of 1.46 and apparent binding constant on G-quadruplex DNA, K_{app}= 9.53 ± 0.08 x 10^4. There was a distinct difference in the binding affinity and selectivity of complexes [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl with apparent binding constant on G-quadruplex DNA, K_{app}= 7.49 ± 0.05 x 10^4 and 9.56 ± 0.07 x 10^4 (Table 4.3). However, for the enantiomer pairs of [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl, the binding affinity are very close to each other with apparent binding constant on G-quadruplex DNA, K_{app}= 9.33 ± 0.03 x 10^4 and 9.53 ± 0.08 x 10^4 (Table 4.4).

The tentative order of DNA binding affinity and selectivity on G-quadruplex DNA of the [Cu(phen)(aa)(H_2O)]X series of complexes is [Cu(phen)(L-threo)(H_2O)]Cl ≈ [Cu(phen)(D-ala)(H_2O)]Cl ≈ [Cu(phen)(L-ala)(H_2O)]Cl > [Cu(phen)(L-threo)(H_2O)]NO_3 ≈ [Cu(phen)(D-threo)(H_2O)]NO_3 > [Cu(phen)(D-threo)(H_2O)]Cl. The result suggested that the [Cu(phen)(aa)(H_2O)]X

complexes that are substituted with different subsidiary ligand possess different extent of binding affinity and selectivity towards G-quadruplex DNA. In this series of complexes, the results indicated that the complex [Cu(phen)(L-threo)(H_2O)]Cl binds strongest and possess the highest selectivity towards G-quadruplex DNA. Conversely, the [Cu(phen)(D-threo)(H_2O)]Cl complex has a poorer affinity and selectivity towards G-quadruplex DNA.

From the result, it is found that the binding affinity and selectivity of the enantiomer pairs, i.e., [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl are very distinct, suggesting that the chirality of subsidiary ligands can play a role in governing the binding affinity and selectivity towards G-quadruplex DNA. Some research groups also reported that chirality is one of the crucial factors for the control of stereospecific and stereoselective interactions (Corrandini *et al.*, 2007). Moreover, Fenton *et al.* (1997) reported that the two different R- and S-enantiomers of platinum(II) complexes, [(R)- and (S)-3-aminohexahydroazepine]dichloro-platinum(II) exhibit diverse binding modes with DNA – an inherently chiral target, with one form of DNA remaining dominantly active over the other. Hence, this should be taken into consideration in designing anti-cancer agent and any artificial G-quadruplex binder.

4.4 Human DNA Topoisomerase I (topo I) Inhibition Study

Topoisomerases (topo) are isomerase enzymes that act on the topology of DNA. DNA topos are ubiquitous and essential enzymes that solve the topological problem accompanying key nuclear processes such as DNA replication, transcription, repair, and chromatin assembly by introducing temporary single- or double-strand breaks in the DNA. These enzymes catalyse the breaking and rejoining of DNA strands. Type I topoisomerase cuts one strand of a DNA double helix, relaxation occurs, and then the cut strand is reannealed. On the other hand, Type II topoisomerase cuts both strands of one DNA double helix, passes another unbroken DNA helix through it, and then reanneals the cut strands (Champoux, 1990, 2001; Wang, 1996, 2002).

Topos control the degree of supercoiling and are required for undoing knots and tangles in the DNA. Such problems are inevitable in eukaryotic nuclei, where several metres of DNA, rotating at high speed during replication, are twisted and folded into a space only a few microns across. The mechanical challenge facing these enzymes is to maintain a firm grip on the two cut ends of one DNA duplex, while folding themselves around the other intact duplex as it passes through the break. Losing hold could be a disaster (Wang, 1991). These enzymes are essential for DNA replication and are important targets for anti viral, anti bacterial and anti tumour drugs (Berger, 1998). In addition, these enzymes fine-tune the steady-state level of DNA supercoiling to facilitate protein

interactions with DNA and to prevent excessive supercoiling that is deleterious (Carey *et al.*, 2003).

Human DNA topoisomerase I (topo I) is a 765-amino acid (aa) (91-kDa) enzyme that catalyzes the relaxation of both negative and positive supercoils in a reaction that does not depend on an energy-rich cofactor or divalent cations (Champoux, 1990). In co-crystal structures of topo I and DNA, the enzyme is tightly clamped around the DNA helix. After cleavage and covalent attachment of the enzyme to the 3'-end at the nick, DNA relaxation requires rotation of the DNA helix downstream of the cleavage site (Carey *et al.*, 2003).

Supercoiled plasmid DNA pBR322 is a suitable substrate for study with topo I, which is a one strand DNA cutter. The supercoiled pBR322 is very compact and moves faster in the gel during electrophoresis. When one strand of the supercoiled DNA is cut, the resultant unwinded, more relaxed open circular pBR322 is formed and this nicked DNA moves slower. When two strands of the supercoiled DNA are cut, the linear DNA is formed and it moves at intermediate speed. The commercial pBR322 (4.4 kb) has a small amount of both more relaxed nicked and linear forms of DNA (Figure 4.44, L2). In the current DNA relaxation assay, one unit of topo I can completely convert all the supercoiled plasmid pBR322 (4.4 kb) to fully relaxed topoisomers, which is the completely unwound covalently bonded closed circular DNA (Figure 4.44, L4).

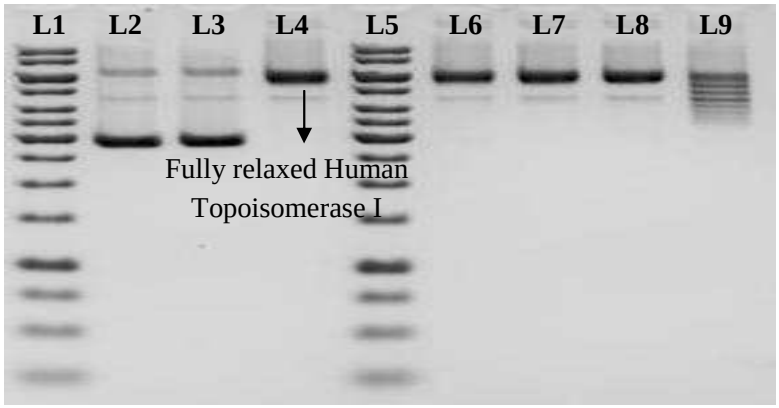

Figure 4.44: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21µL) with pBR322 (0.25 µg) in the absence or presence of 5-40 µM of metal salt, $CuCl_2$. Lane 1 & 5, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 40 µM metal salt (control); Lane 4, DNA + 1 unit topo I (control); Lane 6, DNA + 5 µM metal salt + 1 unit topo I; Lane 7, DNA + 10 µM metal salt + 1 unit topo I; Lane 8, DNA + 20 µM metal salt + 1 unit topo I; Lane 9, DNA + 40 µM metal salt + 1 unit topo I.

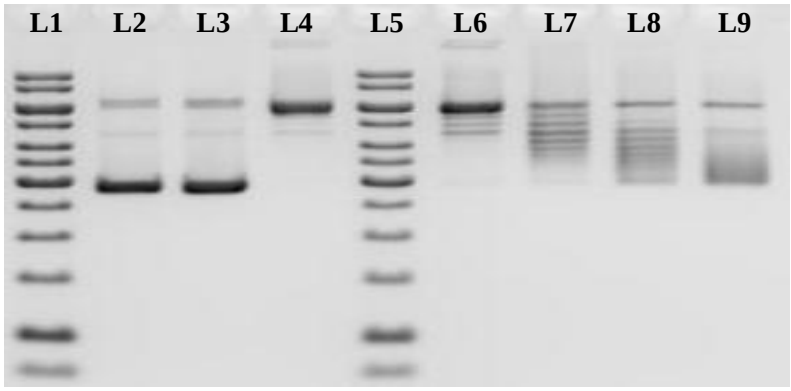

Figure 4.45: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21µL) with pBR322 (0.25 µg) in the absence or presence of 5-40 µM of complex, $[Cu(phen)Cl_2]$. Lane 1 & 5, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 40 µM complex (control); Lane 4, DNA + 1 unit topo I (control); Lane 6, DNA + 5 µM complex + 1 unit topo I; Lane 7, DNA + 10 µM complex + 1 unit topo I; Lane 8, DNA + 20 µM complex + 1 unit topo I; Lane 9, DNA + 40 µM complex + 1 unit topo I.

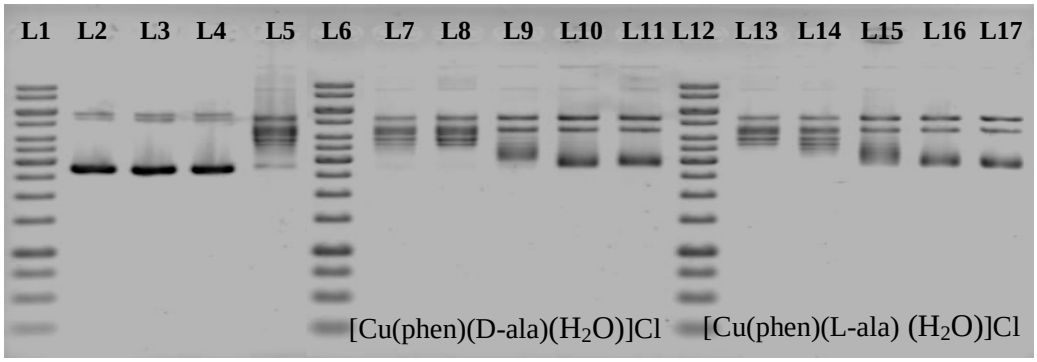

Figure 4.46: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21µL) with pBR322 (0.25 µg) in the absence or presence of 5-200 µM of complex, [Cu(phen)(D-ala)(H_2O)]Cl or [Cu(phen)(L-ala)(H_2O)]Cl. Lane 1, 6 & 12, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 200 µM [Cu(phen)(D-ala)(H_2O)]Cl (control); Lane 4, DNA + 200 µM [Cu(phen)(L-ala)(H_2O)]Cl (control); Lane 5, DNA + 1 unit topo I (control); Lane 7, DNA + 5 µM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I; Lane 8, DNA + 10 µM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I; Lane 9, DNA + 50 µM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I; Lane 10, DNA + 100 µM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I; Lane 11, DNA + 200 µM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I; Lane 13, DNA + 5 µM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I; Lane 14, DNA + 10 µM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I; Lane 15, DNA + 50 µM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I; Lane 16, DNA + 100 µM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I; Lane 17, DNA + 200 µM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I.

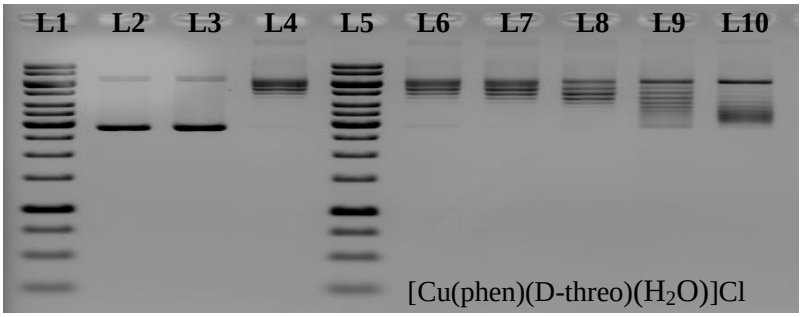

Figure 4.47: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.25 μg) in the absence or presence of 5-200 μM of complex, [Cu(phen)(D-threo)(H_2O)]Cl. Lane 1 & 5, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 200 μM complex (control); Lane 4, DNA + 1 unit topo I (control); Lane 6, DNA + 5 μM complex + 1 unit topo I; Lane 7, DNA + 10 μM complex + 1 unit topo I; Lane 8, DNA + 50 μM complex + 1 unit topo I; Lane 9, DNA + 100 μM complex + 1 unit topo I; Lane 10, DNA + 200 μM complex + 1 unit topo I.

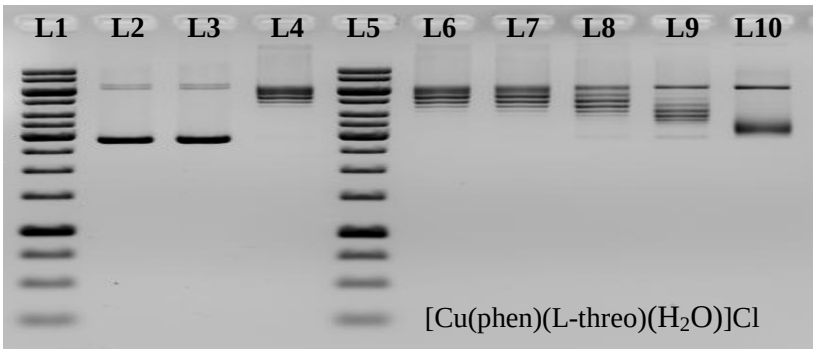

Figure 4.48: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.25 μg) in the absence or presence of 5-200 μM of complex, [Cu(phen)(L-threo)(H_2O)]Cl. Lane 1 & 5, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 200 μM complex (control); Lane 4, DNA + 1 unit topo I (control); Lane 6, DNA + 5 μM complex + 1 unit topo I; Lane 7, DNA + 10 μM complex + 1 unit topo I; Lane 8, DNA + 50 μM complex + 1 unit topo I; Lane 9, DNA + 100 μM complex + 1 unit topo I; Lane 10, DNA + 200 μM complex + 1 unit topo I.

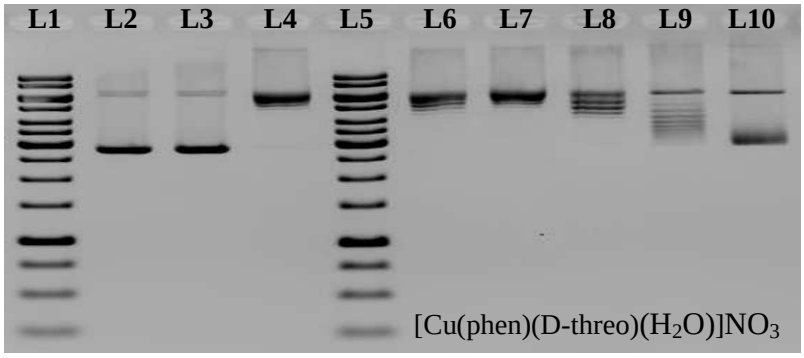

Figure 4.49: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.25 μg) in the absence or presence of 5-200 μM of complex, [Cu(phen)(D-threo)(H_2O)]NO_3. Lane 1 & 5, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 200 μM complex (control); Lane 4, DNA + 1 unit topo I (control); Lane 6, DNA + 5 μM complex + 1 unit topo I; Lane 7, DNA + 10 μM complex + 1 unit topo I; Lane 8, DNA + 50 μM complex + 1 unit topo I; Lane 9, DNA + 100 μM complex + 1 unit topo I; Lane 10, DNA + 200 μM complex + 1 unit topo I.

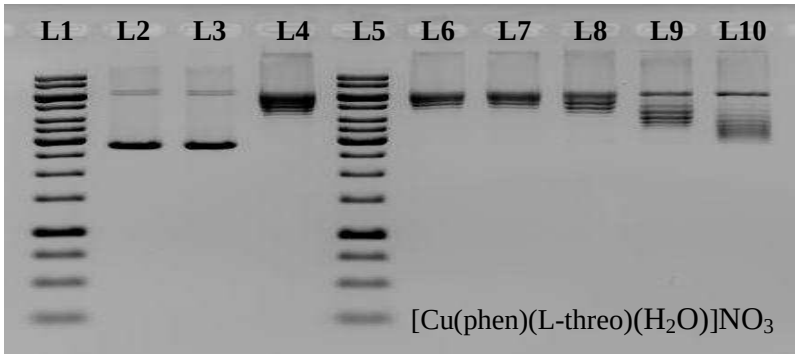

Figure 4.50: Topo I inhibition assay by gel electrophoresis. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.25 μg) in the absence or presence of 5-200 μM of complex, [Cu(phen)(L-threo)(H_2O)]NO_3. Lane 1 & 5, gene ruler 1 Kb DNA ladder; Lane 2, DNA alone; Lane 3, DNA + 200 μM complex (control); Lane 4, DNA + 1 unit topo I (control); Lane 6, DNA + 5 μM complex + 1 unit topo I; Lane 7, DNA + 10 μM complex + 1 unit topo I; Lane 8, DNA + 50 μM complex + 1 unit topo I; Lane 9, DNA + 100 μM complex + 1 unit topo I; Lane 10, DNA + 200 μM complex + 1 unit topo I.

As can be seen from Figure 4.44 (L6 – L9), at low concentration range, 5 – 20 μM, the metal salt, $CuCl_2$, did not inhibit topo I activity, as the DNA bands are the same as those observed for DNA incubated with topo I alone (L4). At higher concentration of $CuCl_2$ (40 μM), high level of inhibition effect of the topo I activity was observed, as some not fully relaxed topoisomers were formed (L9). Interestingly, incubating the pBR322 with topo I and increasing concentration of all the copper(II)-phen-amino acid complexes in this series, viz. [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3, gave rise to the increasing reduction of the nicked band (containing nicked and fully relaxed DNA) and formation of various faster moving bands of topoisomers with different degree of relaxation (Figure 4.46, L7 – L11, L12 – L17; Figure 4.47 – 4.50, L6 – L10). The appearance of faster moving bands of less relaxed topoisomers was observed with increasing concentration of the copper(II) complexes. These results showed that these complexes were able to inhibit the function of topo I in relaxing the supercoiled pBR322, and also showed that the degree of inhibition of copper(II)-phen-amino acid complexes is concentration dependent.

In addition, all six copper(II)-phen-amino acid complexes posses similar extent of inhibition effect on topo I activity, in which the initial inhibitory effect started from 5 μM complex concentration and the inhibitory effect is enhanced with the increasing complex concentration from 5 – 200 μM (Figure 4.46, L7 –

L11, L12 – L17; Figure 4.47 – 4.53, L6 – L10). Surprisingly, for complexes [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, and [Cu(phen)(D-threo)(H_2O)]NO_3, there is almost a total inhibition of topo I activity at the concentration of 200 μM.

The results of the topo I inhibition assay suggested that the topo I activity inhibition effect on plasmid DNA pBR 322 is affected by the type of substituent ligand in a copper(II) complex. For both [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl, a thick supercoiled band can be observed at 200 μM complex concentration (Figure 4.46, L11 & L17). This suggested that both of these copper(II) complexes greatly inhibit the activity of topo I at 200 μM. A thick supercoiled band can be observed at 100 μM for both [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl (Figure 4.46, L10 & L16), while for 100 μM [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl, only topoisomers (but not supercoiled band) can be found (Figure 4.47, L9; Figure 4.48, L9). These results suggested that the alanine-containing copper(II) complexes showed better inhibitory effect compared to those with threonine. Moreover, comparing between pair of enantiomers in each sets of the complexes - [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl, as well as [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3, there are no significant difference in topo I activity inhibition due to the change in chirality of amino acid. Furthermore, the ligand Cl^- and NO_3^- are found to have least or no effect on this

inhibition of topo I activity, as they are suggested to be dissociated from the complex when dissolved in the water and exist as free ions. Nevertheless, Cu^{2+} ion (e.g., exist as $[Cu(H_2O)_6]^{2+}$) cannot inhibit topo I activity but could do so if the coordinated water molecule(s) is/ are replaced with phen and/ or amino acid.

As a preliminary investigation into the mechanism of action of the above topo I inhibition, we used three variations of mixing the DNA, topo I and the copper(II)-phen-amino acid complex (at 50 μM) were used for the topo I inhibition assay.

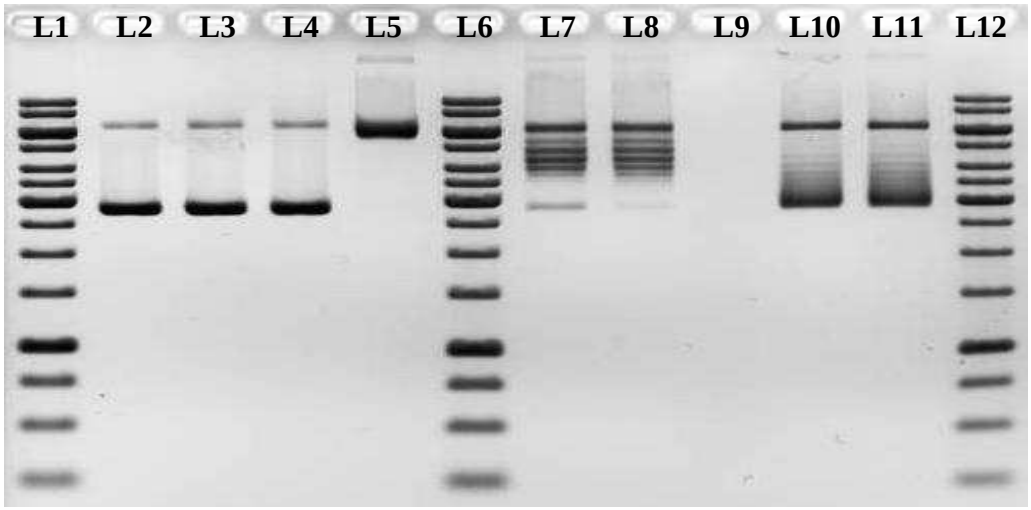

Figure 4.51: Effect of sequence of mixing for the topo I inhibition assay. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.5 μg/μL) and 50 μM copper(II) complexes.

Lane 1, Gene Ruler 1kb DNA ladder
Lane 2, Untreated pBR322 DNA ((0.5 μg/μL) (control)
Lane 3, DNA + 50μM [Cu(phen)(D-ala)(H_2O)]Cl (control)
Lane 4, DNA + 50μM [Cu(phen)(L-ala)(H_2O)]Cl (control)
Lane 5, DNA + 1 unit topo I (control)

[Complex + DNA are incubated for 30 minutes first before topo I is added]

Lane 6, Gene Ruler 1kb DNA ladder
Lane 7, DNA + 50μM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I
Lane 8, DNA + 50μM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I
Lane 9, [Empty]

[Complex + topo I are incubated for 30 minutes first before DNA is added]

Lane 10, DNA + 50μM [Cu(phen)(D-ala)(H_2O)]Cl + 1 unit topo I
Lane 11, DNA + 50μM [Cu(phen)(L-ala)(H_2O)]Cl + 1 unit topo I
Lane 12, Gene Ruler 1kb DNA ladder

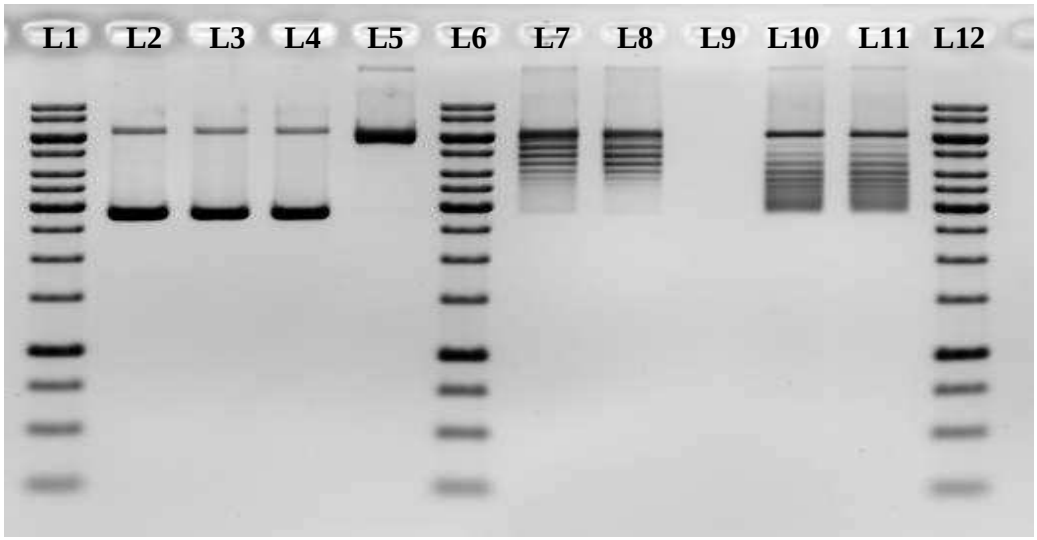

Figure 4.52: Effect of sequence of mixing for the topo I inhibition assay. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.5 μg/μL) and 50 μM copper(II) complexes.

Lane 1, Gene Ruler 1kb DNA ladder

Lane 2, Untreated pBR322 DNA ((0.5 μg/μL) (control)

Lane 3, DNA + 50μM [Cu(phen)(D-threo)(H_2O)]Cl (control)

Lane 4, DNA + 50μM [Cu(phen)(L-threo)(H_2O)]Cl (control)

Lane 5, DNA + 1 unit topo I (control)

[Complex + DNA are incubated for 30 minutes first before topo I is added]

Lane 6, Gene Ruler 1kb DNA ladder

Lane 7, DNA + 50μM [Cu(phen)(D-threo)(H_2O)]Cl + 1 unit topo I

Lane 8, DNA + 50μM [Cu(phen)(L-threo)(H_2O)]Cl + 1 unit topo I

Lane 9, [Empty]

[Complex + topo I are incubated for 30 minutes first before DNA is added]

Lane 10, DNA + 50μM [Cu(phen)(D-threo)(H_2O)]Cl + 1 unit topo I

Lane 11, DNA + 50μM [Cu(phen)(L-threo)(H_2O)]Cl + 1 unit topo I

Lane 12, Gene Ruler 1kb DNA ladder

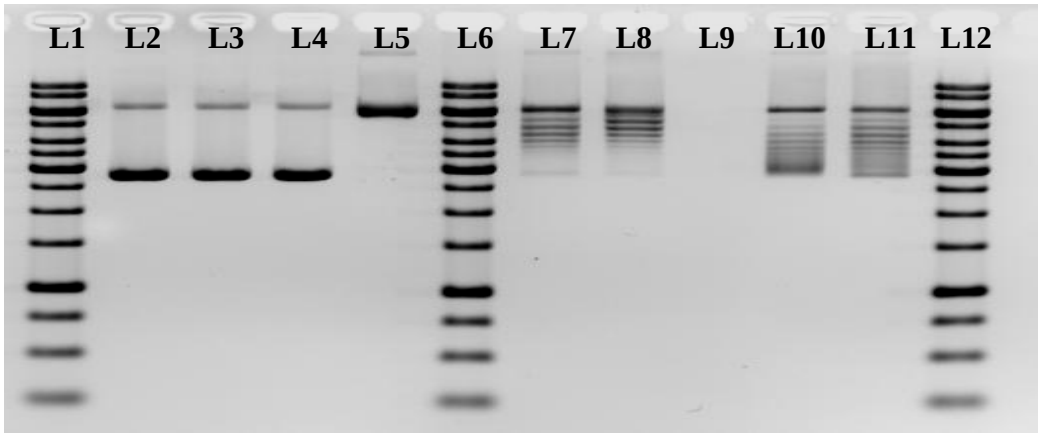

Figure 4.53: Effect of sequence of mixing for the topo I inhibition assay. Electrophoresis results of incubating topo I (1 unit/21μL) with pBR322 (0.5 μg/μL) and 50 μM copper(II) complexes.

Lane 1, Gene Ruler 1kb DNA ladder

Lane 2, Untreated pBR322 DNA ((0.5 μg/μL) (control)

Lane 3, DNA + 50μM [Cu(phen)(D-threo)(H_2O)]NO_3 (control)

Lane 4, DNA + 50μM [Cu(phen)(L-threo)(H_2O)]NO_3 (control)

Lane 5, DNA + 1 unit topo I (control)

[Complex + DNA are incubated for 30 minutes first before topo I is added]

Lane 6, Gene Ruler 1kb DNA ladder

Lane 7, DNA + 50μM [Cu(phen)(D-threo)(H_2O)]NO_3 + 1 unit topo I

Lane 8, DNA + 50μM [Cu(phen)(L-threo)(H_2O)]NO_3 + 1 unit topo I

Lane 9, [Empty]

[Complex + topo I are incubated for 30 minutes first before DNA is added]

Lane 10, DNA + 50μM [Cu(phen)(D-threo)(H_2O)]NO_3 + 1 unit topo I

Lane 11, DNA + 50μM [Cu(phen)(L-threo)(H_2O)]NO_3 + 1 unit topo I

Lane 12, Gene Ruler 1kb DNA ladder

From the gel images presented in Figure 4.51 – 4.53, when the DNA was first incubated with the copper(II)-phen-amino acid complex for 30 minutes before adding in topo I, it can be observed that there was some bands of topoisomers and only a small amount or no supercoiled form of DNA (L7 – L8). These results showed that the inhibitory effect on topo I activity were not strong. When incubating the topo I with the copper(II) complex first before adding DNA, the amount of supercoiled DNA has been increased(Figure 4.51 – 4.53, L10 – L11). The existence of more supercoiled DNA showed that the copper(II) complex possessed higher inhibitory effect if pre-mixing of copper(II) complex with topo I. From the results, it is suggested that the initial binding of copper(II) complexes towards topo I or the DNA may give rise to different mode of action. It is concluded that the inhibitory effect on topo I is greatest when copper(II) complexes interact first with topo I, and the interaction inactivate the topo I from exercising its function.

From the results, both complexes with alanine as subsidiary ligand, i.e., [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl were able to inhibit almost all topo I activity and give rise to a thick band of supercoiled DNA and bands of faster moving, less relaxed topoisomers in the agarose gel image (Figure 4.51, L10 & L11). On the other hand, for complexes with threonine as subsidiary ligand, i.e., [Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3, there are mainly topoisomers (very little supercoiled DNA band) can be found in the gel

image. These results suggested that the subsidiary ligand play a main role in affecting the inhibitory property of copper(II) complexes, and contributing effect of alanine in the topo I inhibitory property of [Cu(phen)(aa)(H_2O)]X is greater than that of threonine.

Interestingly, the inhibitory property of topo I activity between D- and L-enantiomers are found to be different. In this case, complex [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3 (Figure 4.51; Lane 7 & 10, Figure 4.53; Lane 7 & 10) are found to be slightly better compared to [Cu(phen)(L-ala)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]NO_3 (Figure 4.51; Lane 8 & 11, Figure 4.53; Lane 8 & 11), respectively, in inhibiting topo I activity. Again, these results suggested that the substituent ligand does play a role in this topo I activity, even a small difference, i.e. enantiomer of the ligand, may give rise to different extent of topo I inhibitory activity. Nevertheless, changing of amino acid from alanine to threonine of the subsidiary ligands showed a distinct difference. In this case, contributing effect of alanine in the topo I inhibitory property of [Cu(phen)(aa)(H_2O)]X is greater than that of threonine.

Furthermore, the counter anion Cl^- and NO_3^- doesn't give any effect on this inhibitory property of topo I. The anion probably dissociates from the cationic complex when it is dissolved in water and exist as free anion. For example, when both set of copper(II) complexes, [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3, as well as [Cu(phen)(L-threo)(H_2O)]Cl and [Cu(phen)(L-

threo)(H_2O)]NO_3 dissolved in the water, the cations present in the solution are the same (i.e., [Cu(phen)(D-threo)(H_2O)]$^+$ and [Cu(phen)(L-threo)(H_2O)]$^+$, respectively). This explained why the reactivity of these two sets of copper(II) complexes looks very similar to each other (Figure 4.52 – 4.53; L7 – L8, L10 – L11).

CHAPTER 5

CONCLUSION

In this study, the nucleolytic and DNA binding properties of three pairs of ternary copper(II) complexes with 1,10-phenanthroline as main ligand and chiral amino acid (aa) as variable subsidiary ligand were studied by using agarose gel electrolysis, fluorescence quenching assay, restriction enzyme inhibition and human DNA topoisomerase I (topo I) inhibition test. The general formula of this series of complexes can be abbreviated as [Cu(phen)(aa)(H_2O)]X, where aa is amino acid, and X is a chloride or nitrate. The amino acids used were D-alanine, L-alanine, D-threonine and L-threonine.

The experiments were divided into two main parts, viz. nucleolytic study and DNA interaction study. For nucleolytic study, the main objective is to investigate the factors that affect the nucleolytic efficiency of the complexes. The factors studied included complex concentration, types of buffer, pH of buffer, incubation time, and the presence of exogeneous agent (hydrogen peroxide, H_2O_2, or L-ascorbic acid). Interestingly, the type of buffer was found to be one of the factors that affected the DNA cleavage efficiency in this study. All six copper(II) complexes, [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, and

[Cu(phen)(D-threo)(H_2O)]Cl, [Cu(phen)(L-threo)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 showed better cleavage ability in HEPES buffer. They were able to convert supercoiled DNA into nicked and/ or linear form of DNA in Hepes buffer.

The pH of the buffer was also found to affect the DNA cleavage efficiency of the complex. [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]Cl, and [Cu(phen)(L-threo)(H_2O)]Cl showed slightly better cleavage efficiency in TN buffer at basic pH (pH 7.2 or pH 8.2) than acid pH (pH 6.2) while [Cu(phen)(D-threo)(H_2O)]NO_3 and [Cu(phen)(L-threo)(H_2O)]NO_3 complexes were found to be slightly better at pH 6.2 in TN buffer. The DNA cleavage efficiency of the complex was found to increase with increasing incubation time. [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl showed slightly better cleavage compared to others. After incubation with DNA for 48 hours, both were able to convert almost all of the supercoiled DNA into nicked and linear from of DNA. The presence of exogeneous (either hydrogen peroxide or L-ascorbic acid) enhanced the nucleolytic efficiency of the complexes. However, the cleavage efficiency between pairs of enantiomer (D- and L-) are very similar in the presence of either hydrogen peroxide or L-ascorbic acid.

For DNA interaction study, the aim is to investigate binding selectivity or specificity of the copper(II) complexes towards different types and different

sequences of DNA. Types of DNA that were used in this part were calf thymus (CT) DNA, G-quadruplex DNA (*telo21*), and oligonucleotides with different sequences, viz. ds$(AT)_6$ and ds$(CG)_6$. The complexes [Cu(phen)(D-ala)(H_2O)]Cl, [Cu(phen)(L-ala)(H_2O)]Cl, [Cu(phen)(D-threo)(H_2O)]NO_3, and [Cu(phen)(L-threo)(H_2O)]NO_3 binds preferentially or more selectively to ds$(AT)_6$ than ds$(CG)_6$. The [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]Cl seemed to have no preference as their binding constants for both ds$(AT)_6$ and ds$(CG)_6$ were very close. The complex [Cu(phen)(L-ala)(H_2O)]Cl bound most strongly to ds$(AT)_6$ oligonucleotides as its apparent binding constant ($8.26 \pm 0.16 \times 10^4$ M^{-1}) is distinctly higher than those of other five of these copper(II) complexes.

The binding selectivity is higher if the complex inhibits lesser restriction enzymes. A non-selective, random complex will inhibit all the twelve restriction enzymes used. All six complexes were able to inhibit 2 – 4 of the twelve restriction enzymes. In this series of complexes, [Cu(phen)(L-ala)(H_2O)]Cl inhibited the four restriction enzymes, viz. Ase I, Ssp I, Nde I, and Bst 11071 while [Cu(phen)(D-ala)(H_2O)]Cl inhibited two, viz. Ssp I and Nde I. [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3 inhibited Ssp I, Nde I and Bst 11071 while [Cu(phen)(L-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]NO_3 inhibited Ssp I and Nde I. Thus, the DNA binding of [Cu(phen)(D-ala)(H_2O)]Cl is more selective than that of [Cu(phen)(L-ala)(H_2O)]Cl while the DNA binding of both [Cu(phen)(L-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]NO_3 are more selective than those of [Cu(phen)(D-

threo)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3. These results show that both chirality and the type of amino acid influence the DNA binding selectivity of the chiral [Cu(phen)(aa)(H_2O)]X complexes. Interestingly, [Cu(phen)(L-ala)(H_2O)]Cl has the least DNA binding selectivity as it inhibited four restriction enzymes while the other complexes inhibited two or three. We are still unclear about the cause of the differences or the similarities.

The human DNA topoisomerase I (topo I) inhibition assay suggested that the topo I activity inhibition effect on plasmid DNA pBR 322 is affected by the type of subsidiary ligand in a copper(II) complex. From the results, both 200 μM of both [Cu(phen)(D-ala)(H_2O)]Cl and [Cu(phen)(L-ala)(H_2O)]Cl could inhibit almost all topo I activity and gave rise to a thick band of supercoiled DNA in the agarose gel image. The topo I inhibition of the pairs of [Cu(phen)(D-threo)(H_2O)]Cl and [Cu(phen)(D-threo)(H_2O)]NO_3, as well as [Cu(phen)(L-threo)(H_2O)]Cl and [Cu(phen)(L-threo)(H_2O)]NO_3 were the same. The counter anion, Cl^- and NO_3^- might dissociate from the complex and produced the same cationic species, [Cu(phen)(threo)(H_2O)]$^+$ when they dissolved in water, hence, we can conclude that the counter anion Cl^- and NO_3^- doesn't give to any effect on this inhibitory of topo I.

Last but not least, according to the apparent binding constants and 22G/17bp ratio obtained through the thiazole orange displacement assay, the order of G-quadruplex DNA binding affinity and selectivity of

[Cu(phen)(aa)(H_2O)]X series of complexes is [Cu(phen)(L-threo)(H_2O)]Cl ≈ [Cu(phen)(D-ala)(H_2O)]Cl ≈ [Cu(phen)(L-ala)(H_2O)]Cl > [Cu(phen)(L-threo)(H_2O)]NO_3 ≈ [Cu(phen)(D-threo)(H_2O)]NO_3 > [Cu(phen)(D-threo)(H_2O)]Cl, with the ratio of quadruplex DNA apparent binding constant 22G over duplex DNA apparent binding constant 17bp, Ratio 22G/ 17bp of 1.49, 1.47, 1.46, 1.25, 1.24, and 1.18, respectively. These results show that the chirality of subsidiary ligands could affect the binding affinity and selectivity towards G-quadruplex DNA.

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APPENDICES

APPENDIX A

The recipe for the preparation of buffers and solutions

The recipe for the preparation of 50X Tris-Acetate-EDTA electrophoresis buffer stock solution.

1. The following chemicals were dissolved in 800 ml of sterile distilled water.
   242 g Tris Base (Formula Weight = 121.14)
   57.1 ml glacial acetic acid
   100 ml 0.5 M EDTA, pH 8.0
2. Final volume was adjusted to 1 liter with sterile distilled water. Mix thoroughly.
3. The buffer was stored indefinitely at room temperature.

The recipe for the preparation of Phosphate buffer, pH 7.5.

1. The following chemicals were dissolved in 800 ml of sterile distilled water.
   0.5193 g Sodium dihydrogen
   4.3516 g Disodium hydrogen
   1.7532 g Sodium Chloride (NaCl)
2. Final volume was adjusted to 1 liter with sterile distilled water. Mix thoroughly.
3. pH was adjusted to pH 7.5 by using Hydrochloric Acid (HCl) or Sodium Hydroxide (NaOH).
4. The buffer was stored indefinitely at room temperature.

The recipe for the preparation of Hepes buffer, pH 7.5.

1. The following chemicals were dissolved in 800 ml of sterile distilled water.
   5.6662 g/L Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
   1.7532 g/L Sodium Chloride (NaCl)
2. Final volume was adjusted to 1 liter with sterile distilled water. Mix thoroughly.
3. pH was adjusted to pH 7.5 by using Hydrochloric Acid (HCl) or Sodium Hydroxide (NaOH).
4. The buffer was stored indefinitely at room temperature.

The recipe for the preparation of 0.5 mM H_2O_2.

1. 2.1 µl of standardized H_2O_2 solution is transferred to an Eppendorf tube and top up with 997.9 µl of sterile distilled water. The concentration of this stock solution is 20 mM.
2. Dilution been carried out to dilute the stock solution to concentration of 0.5 mM.
3. 25 µl of H_2O_2 has been transferred from stock solution to new Eppendorf tube and top up with 975 µl of sterile distilled water.

The recipe for the preparation of 10 µM Ascorbic Acid (AA).

1. Ascorbic Acid in powder forms were weight in the range of 1.0 – 1.5 mg.
2. Volume of sterile distilled water needed for top up purposes to prepare 10mM stock solution is calculated (shown in Appendix C).
3. Serial dilution been carried out to dilute the stock solution to concentration of 10 µM.
4. 100 µl of AA has been transferred from stock solution to new Eppendorf tube and top up with 900 µl of sterile distilled water. 10 µl has been transferred out from this mixture, to the new Eppendorf tube, and top up with 990 µl of sterile distilled water.

APPENDIX B

Protocol for preparation, stain, and view of DNA samples.

1. Plasmid DNA, pBR 322 is pipetted out and transferred to a sterile Eppendorf tube (autoclaved).
2. Addition of buffer, complex, exogenous agent (AA or H_2O_2) into the tube followed the sequence as stated above.
3. Samples have been spin by centrifuge machine at 1500 rpm for 1 minute.
4. Samples were sending for incubation.
5. After incubation, samples were get out from the incubator and spin at 1500 rpm for 1 minute.
6. The samples were quenched by addition of 3 µL of 6X loading dye.
7. The samples were spin at 1500 rpm for 1 minute.
8. Samples were loaded into 1.25 % agarose gel, and run at 80 V for 1 hour.
9. The agarose gel for each experiment were stained with 0.5 µg/ µL ethidium bromide for 30 minutes, then visualized by using UV transilluminator and photographed by using GeneFlash programme.

APPENDIX C

The calculation in the preparation of buffers and solutions

The calculation to get volume of buffer/ solution needed for top up purposes.

$$\text{Volume of Buffer/ Solution} = \frac{\text{Weight (mg)}}{\frac{\text{Molecular Weight}}{\text{Molarity (M)}}}$$

i.e., Complex A weight = 1.5 mg

Molecular weight of complex A = 198.11 g/ mol

Molarity needed = 0.01 M

$$\text{Volume of Buffer/ Solution} = \frac{1.5\text{mg}}{\frac{198.11}{0.01\text{M}}}$$

$$= 0.757$$

Hence, 757 µl of buffer/ solution needed to prepare 0.01M of complex A.

The calculation for dilution purposes.

$$M_1V_1 = M_2V_2$$

M_1 = Molarity of stock solution

V_1 = Volume of stock solution needed for dilution

M_2 = Molarity needed (after dilution)

V_2 = Volume of diluted solution needed

i.e.,

M_1 = 10 M

V_1 = X

M_2 = 0.01 M

V_2 = 1000 µl

$M_1V_1 = M_2V_2$

$(10)(X) = (0.01)(1000)$

$X = 10/10$

$= 1$

Hence, 1 µl of stock solution needed to top up with 999 µl distilled water or buffer to get 0.01 M diluted solution.