SYNTHESIS, CHARACTERIZATION AND DNA CLEAVAGE ABILITY OF COBALT(II) AND ZINC(II) COMPLEXES

by

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LIST OF ABBREVIATIONS

AAS = Atomic absorption spectroscopy

CHN = Carbon, hydrogen, nitrogen

Co(2-pc) = diaquabis(picolinato)cobalt(II)dihydrate

Co(3,5-dn) = tetraaquabis(3,5-dinitrobenzoato)cobalt(II)tetrahydrate

Co(4-amino) = diaquabis(4-aminobenzoato)cobalt(II)

Co(gly) = diaquadichloro(glycine)cobalt(II)

Co(glycol) = bis(glycolato)cobalt(II)

Co(mal) = diaquabis(malonato)cobalt(II)

Co(maleic) = tetraaquabis(maleato)cobalt(II)

Co(p-2c) = diaquabis(pyrazine-2-carboxylato)cobalt(II)

Co(pyd-2,5-dc) = diaquabis(pyridine-2,5-dicarboxylato)cobalt(II)

Co(quino) = diaquabis(pyridine-2,3-dicarboxylato)cobalt(II)

$CoCl_2 \cdot 6H_2O$ = cobalt(II)chloride hexahydrate

CV = Cyclic voltammetry

DMSO = dimethyl sulfoxide

DNA = Deoxyribonucleic acid

FT-IR = Fourier transform infrared spectroscopy

H_2O_2 = hydrogen peroxide

KI = potassium iodide

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaN_3 = sodium azide

ROS = Reactive oxygen species

TGA = Thermogravimetric analysis

UV-Vis = Ultraviolet-visible spectrometry

Zn(2-pc) = diaquabis(picolinato)zinc(II)dihydrate

$Zn(CH_3COO)_2 \cdot 2H_2O$ = zinc(II)acetate dihydrate

Zn(mal) = diaquabis(malonato)zinc(II)

Zn(maleic) = tetraaquabis(maleato)zinc(II)

Zn(p-2c) = diaquabis(pyrazine-2-carboxylato)zinc(II)

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SINTESIS, PENCIRIAN DAN DAYA PEMBELAHAN DNA KOMPLEKS KOBALT(II) DAN KOMPLEKS ZINK(II)

ABSTRAK

Sebanyak sepuluh kompleks kobalt(II) dan emapt kompleks zink(II) telah berjaya disintesiskan daripada tindak balas antara kobalt(II)klorida heksahidrat, $CoCl_2 \cdot 6H_2O$ atau zink(II)asetat dihidrat, $Zn(CH_3COO)_2 \cdot 2H_2O$ dengan ligan asid karboksilik. Lapan kompleks yang mempunyai isostruktur yang sama telah disintesiskan antara $CoCl_2 \cdot 6H_2O$ atau $Zn(CH_3COO)_2 \cdot 2H_2O$ dengan ligan asid pirazina-2-karboksilik, asid maleik, asid 2-pikolinik dan asid malonik. Seterusnya, enam kompleks yang lain telah disintesis melalui $CoCl_2 \cdot 6H_2O$ dengan ligan glisina, asid glikolik, asid 3,5-dinitrobenzoik, asid quinolinik, asid piridina-2,5-dikarboksilik dan asid 4-aminobenzoik. Kompleks yang telah disintesiskan ini dicirikan melalui pelbagai kaedah fizik seperti penentuan takat lebur, konduktiviti, Spektroskopi Inframerah (FT-IR), Mikroanalisis CHN, Spektroskopi Penyerapan Atom (AAS), Spektroskopi Ultralembayung (UV-Vis), Voltametri Siklik (CV), Analisis Termogravimetrik (TGA) dan X-ray Kristralografi. Manakala sifat biologi kompleks ini telah dianalisis melalui Pemotongan Plasmid DNA dan Pengasaian MTT. Melalui analisis Spektroskopi Inframerah, didapati bahawa kesemua 14 kompleks ini menunjukkan ligan terikat secara monodentat kepada pusat atom Co(II) atau Zn(II). Manakala, analisis UV-Vis menunjukkan bahawa keadaan pengoksidaan bagi atom kobalt dalam kompleks ialah +2 dan mempunyai konfigurasi d^7 dan kompleks zink juga dalam keadaan pengoksidaan + 2 tetapi dengan konfigurasi d^{10}. Keupayaan sifat redoks di dalam kompleks adalah amat perlu untuk kegunaan biologi dan ini dapat

ditentukan melalui analisis CV. Hasil kajian eksperimen pemotongan plasmid DNA menunjukkan kesemua kompleks berupaya memotong plasmid DNA dimana pemotongan tersebut menghasilkan jaluran tunggal serta jaluran berganda DNA. Keberkesanan pemotongan plasmid DNA ini bergantung secara langsung dengan kepekatan kompleks. Kompleks kobalt(II) menunjukkan pemotongan plasmid yang lebih baik berbanding dengan kompleks zink(II). Selain itu, kompleks kobalt(II) juga memberikan keputusan aktiviti sitotoksik yang lebih baik berbanding dengan kompleks zink(II) dalam eksperimen pengasaian MTT.

SYNTESIS, CHARACTERIZATION AND DNA CLEAVAGE ABILITY OF COBALT(II) COMPLEXES AND ZINC(II) COMPLEXES

ABSTRACT

A total of ten cobalt(II) complexes and four zinc(II) complexes had been successfully synthesized between the reaction of cobalt(II)chloride hexahydrate, $CoCl_2 \cdot 6H_2O$ or zinc(II)acetate dihydrate, $Zn(CH_3COO)_2 \cdot 2H_2O$ with carboxylic acids. Eight isostructural of complexes Co(II) and Zn(II) were formed by the reaction of $CoCl_2 \cdot 6H_2O$ or $Zn(CH_3COO)_2 \cdot 2H_2O$ with pyrazine-2-carboxylic acid, maleic acid, 2-picolinic acid and malonic acid while the others six complexes were synthesized by the reaction of $CoCl_2 \cdot 6H_2O$ with glycine, glycolic acid, 3,-5dinitrobenzoic acid, quinolinic acid, pyridine-2,5-dicarboxylic acid and 4-aminobenzoic acid. These complexes were characterized by determination of melting point, conductivity measurement, Fourier Transform Infrared Spectroscopy (FT-IR), microanalysis CHN, Atomic Absorption Spectroscopy (AAS), Cyclic Voltammetry (CV), Thermogravimetry Analysis (TGA) and X-ray Crystallograhy. While the biological properties of these complexes were determined by DNA Cleavage and MTT Assay. From the FT-IR analysis, all the ligands coordinated the atoms Co(II) and Zn(II) in monodentate. While from UV-Vis analysis, the oxidation state of the cobalt(II) complexes were in +2 and showed d-d transition peaks corresponding to d^7 system while the oxidation state of zinc(II) complexes were also +2 and in d^{10} system. The CV analysis showed that all these complexes have the reversible redoxs ability which is very important in the biological uses. Gel electrophoresis experiments showed that all the complexes successfully promoted the cleavage of

plasmid DNA by producing single or double DNA strand breaks. All the cobalt(II) complexes were shown better in biological properties compared to zinc(II) complexes whether in DNA cleavage or MTT Assay.

CHAPTER 1

INTRODUCTION

1.1 DNA

Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses (Figure 1.1).

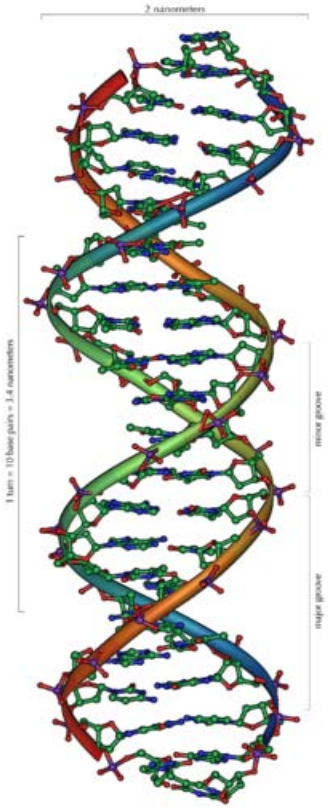

Figure 1.1 Double helix DNA

The main role of DNA molecules is the long-term storage of information. DNA is often compared to a set of blueprints or a recipe, since it contains the

instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information.

Chemically, DNA consists of two long polymers of simple units called nucleotides, with backbones made of sugars and phosphate groups joined by ester bonds. These two strands run in opposite directions to each other and are therefore anti-parallel. Attached to each sugar is one of four types of molecules called bases (adenine, thymine, guanine and cytosine) (Figure 1.2).

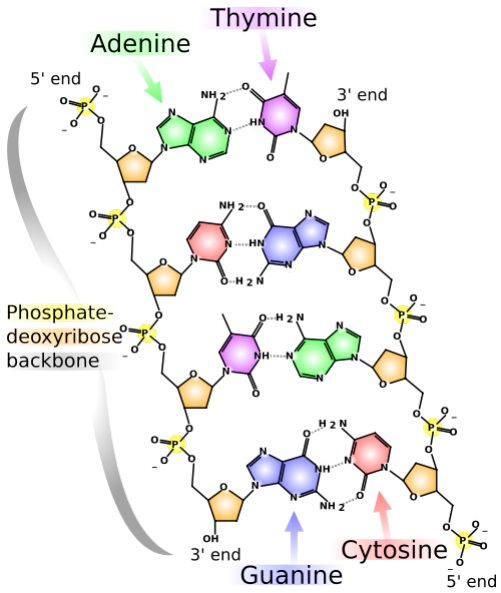

Figure 1.2 The chemical structure of DNA

It is the sequence of these four bases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the nucleic acid RNA, in a process called transcription.

Within cells, DNA is organized into structures called chromosomes. These chromosomes are duplicated before cells divide, in a process called DNA replication. Eukaryotic organisms (animals, plants, fungi, and protists) store their DNA inside the cell nucleus, while in prokaryotes (bacteria and archae) it is found in the cell's cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA. These compact structures guide the interactions between DNA and other proteins, helping control which parts of the DNA are transcribed.

1.2 Metals used as DNA cleaving agents

According to the American Cancer Society, cancer is the second leading cause of death in the United States. For this reason, over the past 40 years, there has been a vast interest in development of efficient anticancer drugs that possess minimal side effects. Cancer is a class of disease in which there is uncontrolled cell division. Maligmant tumor cells spread directly into the adjacent tissue or indirectly by metatasis. Therefore, the interaction of transition metal complexes with DNA has been extensively studied in the past few decades. Beginning in the early 1980’s, researchers began examining the interactions of coordinated complexes of cobalt, zinc, copper, nickel, iron, chromium, rhodium, osmium and ruthenium. DNA-interactions of many transition metal compounds containing intercalative ligands such as 1,10-phenanthroline and substituted phenanthrolines had been investigated. Metal complexes are a particularly attractive species to recognize and cleave DNA. The ligands or the metal in these complexes can be varied in an easily controlled manner to facilitate an individual application. The change in metal ion or ligand

leads to changes in the binding mode and affinity. Transition metal complexes can interact non-covalently with DNA by intercalation, groove binding, or external electrostatic binding. Among the factors governing the binding modes, it appears that the most significant is the molecular shape. Those complexes that best fit against the DNA helical structure display the highest binding affinity. Many useful applications of these complexes require that the complex bind to DNA through an intercalative mode with the ligand intercalating into the adjacent base pairs of DNA. When the size, shape and chirality of the complexes fit the DNA structure, the complexes may approach closely to and intercalate into the base pairs of DNA. On the other hand, upon irradiation, these complexes can induce the single or double-stranded cleavage of plasmid DNA. Although the interactions of the complexes with DNA have been investigated extensively, the knowledge of the nature of binding of some complexes and their binding geometries has remained relatively modest.

One of the usage of transition metal complexes is anticancer agents. Disruption of DNA synthesis in cancerous cells is carried out by transition metal complexes in a variety of ways. For example, titanium uses the glyoprotein transferrin to enter the cell, gold is highly cytotoxic, attacking the mitochondria but it also attacking healthy cells, estrogen–derived steroidal metal complexes access the cells by binding to estrogen receptors. Palladium acts by disruption of the double helix in the DNA leaving most of the base pairs intact indicating that the complex does not insert itself into the sequence.

The first transition metal successfully used as an anticancer agent was platinum. It was used in a compound called cisplatin (Figure 1.3) (Rosenberg *et al*.,1969; Rosenberg *et al*., 1970).

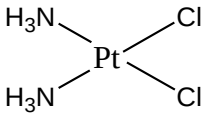

Figure 1.3 Cisplatin

Platinum complexes form interstrand and intrastrand adducts depending on the number of metal centres. This enables high mobility group (HMG1) proteins to recognize damaged DNA (Cox *et al*, 2001). It also reacts with neighboring purine residues (adjacent intrastand guanines and adenines) to form intrastand adducts in DNA (Sullivan *et al*, 2000). So, it has the ability to inhibit tumors and is widely used alone or in combination with other chemotherapeutic drugs for the treatment of several aggressive cancers like solid malignant tumors such as those found in the head and neck, lungs, ovary, bladder and testicles (Abrams and Messer, 1993: Weiss and Christian, 1993). Although treatment with cisplatin is offen effective, serious side-effects such as nausea, nephrotoxicity, neurotoxicity and ototoxicity often occur (Reedijk, 1996). In addition, cisplatin, which is administered intravenously, has limited solubility in aqueous solution (Wong and Giandomenico, 1999). This has lead to intensive efforts in developing newer platinum drugs that show similar efficiency to cisplatin but exhibit less toxicity.

1.3 Usage and application of cobalt complexes

Cobalt has both beneficial and harmful effects on humans. Cobalt is an essential element for humans since it is part of vitamin B12. The amount needed is very small, and the body contains only about 1 milligram. It may be used in the

treatment of anemia. Exposure to cobalt may cause lung effects, which include respiratory irritation, coughing, asthma, pulmonary oedema, and pneumonia. The International Agency for Research on Cancer classifies cobalt as a 'possible human carcinogen'. Exposure to cobalt salts may cause skin problems, and may damage the kidneys and cause lung damage. Cobalt can also damage the heart, causing heart failure. Repeated exposure can cause scarring of the lungs (pulmonary fibrosis) which may not be noticed without a chest x - ray. This can be disabling or fatal.

The largest use of cobalt metal is to make alloys, which retain strength even when very hot. It is also used to help paint dry quickly. Cobalt is also used to make artificial body parts such as hip and knee joints. Cobalt carbonate is used in ceramics and as an animal feed supplement in trace amounts. Cobalt chloride is used as a humidity and water indicator, in electroplating, in the manufacture of vitamin B-12, as a fertilizer and feed additive in trace amounts. Cobalt oxide is used in pigments for ceramics and glass, in fast drying paints and varnishes, in semiconductors, in enamel coatings on steel, and again as an animal feed additive in trace amounts. Cobalt sulfate is used in electroplating, in batteries, as a drying agent in inks and varnishes, in enamels, and ceramics, and as a feed and fertilizer additive.

1.4 Cobalt in antitumor complexes

Mer-tris(β-alaninato)cobalt(III), *mer*-[Co(β-ala)$_3$] alone does not cleave DNA but can do so in the presence of ascorbic acid in TBE buffer pH 8.3. DNA cleavage can be attributed to ascorbate free radicals generated by one-electron transfer from ascorbate ions to *mer*-[Co(β-ala)$_3$]. The nucleolytic ability of *mer*-[Co(β-ala)$_3$] in the

presence of ascorbic acid results from the reduction of the complex by ascorbic acid. Using MTT assay, preliminary studies revealed anti-cancer properties of $CoCl_2$ (IC_{50}=0.06 mM), *mer*-[Co(β-ala)$_3$] (IC_{50}=2.06 mM) and {*mer*-[Co(β-ala)$_3$]+0.20 mM H_2A system} (IC_{50}=1.61 mM) towards lung cancer cell line PC9 (Ng *et al*, 2005).

Bis(phenanthrenequinoato)cobalt have been synthesized and tested on the human breast cancer cell line T47D rich in progesterone receptors and the IC_{50} value is 2.04 µM (Afrasiabi *et al*, 2003).It is clearly observed that complexation with metals has a synergetic effect on the antiproliferative activity. Under certain conditions, a number of metals have been observed to inhibit steroid binding to the cognate receptors or prevent dimerization of the estrogen receptors, due perhaps to the involvement of the thiols in steroid binding.

Generally, cobalt (II) and cobalt (III) complexes show antitumor activities *in vivo*. Hence, recently the interaction of cobalt complexes with DNA has attracted much attention. In a report, Shimakoshi et al.,2003 have shown that a dicobalt complex was more efficient in cleaving DNA compared to an equivalent monomeric complex. Synthesis of new cobalt (II) and cobalt (III) complexes enabled chemists to extensively study the ability of these complexes to act as probes in investigating the structure of DNA. One compound is *cis*-[Co(phen)2(BPEI)Cl]$Cl_2^{-}\cdot 4H_2O$ (phen=1,10-phenanthroline, BPEI=branched polyethyleneimine) (Shimakoshi *et al*, 2003). Preliminary results from the absorption and fluorescence spectral studies show that the polymer–cobalt(III) complex with high cobalt(III) chelate content binds strongly to DNA. Binding of this complex with DNA was also studied by gel electrophoresis using plasmid pBR 322 DNA. This DNA moves on agarose gel under the influence of an electrical field. This movement is retarded when the DNA are bound to other molecules. Many polycationic polymeric molecules interact with DNA through

electrostatic interactions between phosphate groups of the DNA and oppositely charged groups of polymer. The gel retardation assay performed on pBR322 DNA demonstrates that the cationic segments of the polyethyleneimine might have neutralized the negative charges of DNA which could have facilitated further due to the increased intercalation of the planar phen groups of cobalt(III) complex moieties. The IC_{50} value of the complex was higher for the 24 h treatment groups, i.e., in the range of 100–140 µg/mL, whereas for the 48 h treatment groups the IC_{50} value fell in the range of 45–65 µg/mL.

1.5 Usage and application of zinc complexes

Zinc is also an essential metal for human health. It is vital for many biological functions such as disease resistance, wound healing, digestion, reproduction, physical growth, diabetes control, taste and smell. It is estimated that 3000 of the hundreds of thousands of proteins in the human body contain zinc prosthetic groups. In addition, there are over a dozen types of cells in the human body that secrete zinc ions, and the roles of these secreted zinc signals in medicine and health are now being actively studied. Intriguingly, brain cells in the mammalian forebrain are one type of cell that secretes zinc, along with its other neuronal messenger substances. Cells in the salivary gland, prostate, immune system and intestine are other types that secrete zinc.

Zinc is an activator of certain enzymes, such as carbonic anhydrase. Carbonic anhydrase is important in the transport of carbon dioxide in vertebrate blood. It is

also required in plants for leaf formation, the synthesis of indole acetic acid (auxin) and anaerobic respiration (alcoholic fermentation).

1.6 Zinc in antitumor complexes

Zn(phen)(edda) (phen=1,10-phenanthroline, edda=ethylenediaminediacetic acid) complexes was found to have promising antitumor qualities. The gel electrophoresis result shows that the extent of DNA cleavage by the Zn(phen)(edda) complex is dependent on both the concentration. The concentration factor has a limiting effect as no further increase in DNA cleavage occurs beyond the limiting concentration. The IC_{50} for this compound is 7.0 µg/mL for incubation time of 72 hours (Seng *et al*, 2008). Tatjana *et al.* showed that the antiproliferative activity of zinc(II) complexes $[Zn(AcPipPheF)_2]$ and $[Zn(OAc)(AcPip\text{-}PheF)]_2$ where AcPip-PheF is 2-acetyl pyridine 1-(4-fluorophenyl)-piperazinyl thiosemicarbazone was found to be considerably stronger than that of cis-platin. The IC_{50} values range from 26 to 90 nM, against all cell lines tested, while for cis-platin the IC_{50} values range from 2 to 17 µM and for the zinc salt, $ZnCl_2$, the IC_{50} values range from 81 to 93 µM (Tatjana *et al*, 2010). Zn(II) complexes $[ZnCl_2(Fo4Npypipe)]$ and $[ZnCl_2(Ac4Npypipe)]$ where Fo4Npypipe and Ac4Npypipe are 2-formyl pyridine N(4)-1-(2-pyridyl)-piperazinyl thiosemicarbazoneare and 2-acetyl pyridine N(4)-1-(2-pyridyl)-piperazinyl thiosemicarbazone respectively considered as agents with potential antitumor activity, and can therefore be candidates for further stages of screening *in vitro* and/or *in vivo* (Dimitra *et al*, 2006).

1.7 Mechanism of DNA cleavage

DNA damage is well known to play a critical role in many diseases. Therefore, most metal-based drugs on the market require dioxygen to bring about DNA damage. Even fewer have been shown to induce damage *via* carbon centered radicals. These are the two pathways.

1.7.1 Reactive oxygen species (ROS)

The enzyme superoxide dismutase is able to dismutate superoxide anion to form oxygen and hydrogen peroxide. Those ROS have been found to interact directly with DNA or DNA components *via* singlet oxygen species ($O_2^{\cdot-}$) and hydroxide radicals ($^{\cdot}OH$). Under aerobic conditions, react with oxygen to produce $O_2^{\cdot-}$ (Heo *et al*, 2006). However, several works in literature have shown that $O_2^{\cdot-}$ radicals cannot induce DNA damage (Muller and Burrows, 1998). These $O_2^{\cdot-}$ can react with H^+ to generate H_2O_2 which in turn can react with more $O_2^{\cdot-}$ to generate more $^{\cdot}OH$ *via* Harber-Weiss reactions. The redox potential for $^{\cdot}OH$ radicals is E≈2.4 V vs. NHE. Since the redox potential for $^{\cdot}OH$ is high, $^{\cdot}OH$ can oxidize DNA, resulting in DNA damage.

ROS induce oxidative stress. *In vivo*, radicals are thought to be produced *via* two main pathways. The first is *via* the exposure of living organisms to ionizing radiation (γ radiolysis) leading to the hemolytic fission of oxygen-hydrogen bonds in water thereby producing an $^{\cdot}OH$. The second way is through the generation of $^{\cdot}OH$ through Fenton reactions. These reactions usually involve a metal ion which acts as a

catalyst in the presence of hydrogen peroxide. The hydrogen peroxide is broken down into a hydrogen ion and a hydroxyl free radical (Figure 1.4).

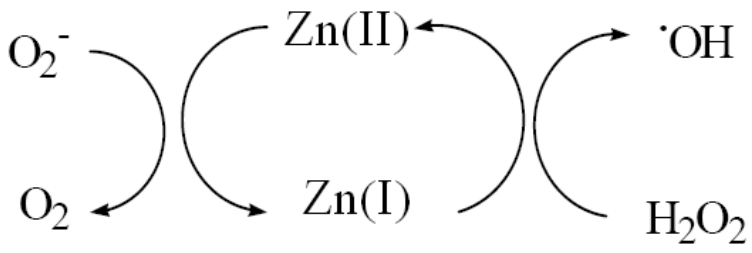

Figure 1.4 Schematic diagram showing metal ion conversion to hydroxyl radicals

It has been shown that DNA ·OH radicals induce DNA damage *via* hydrogen abstraction from the sugar moiety hence forming sugar radicals (Tullius and Pogozelski, 1998). Proven that the major product formed by ·OH DNA damage was 8-OH-Gua suggesting that ·OH radicals are involved in the attack of purine bases (Floyd *et al,* 1986). Three major intermediates that result from ·OH radical attack had been identified. The chemical fates of these intermediates have been extensively studied. Adduct 1 and 2 revert back to guanine by gaining an electron *via* thiols generated in the cells. Adduct 3 (8-OH-Gua) leads to the formation of 7,8-dihydro-8-oxoguanine or can gain an electron and a proton forming 2,6-diamino-5-formamido-4-hydroxypyrimidine (FAPy-G) which eventually leads to DNA damage (Figure 1.5) (Steenken, 1989; O'Neill, 1983).

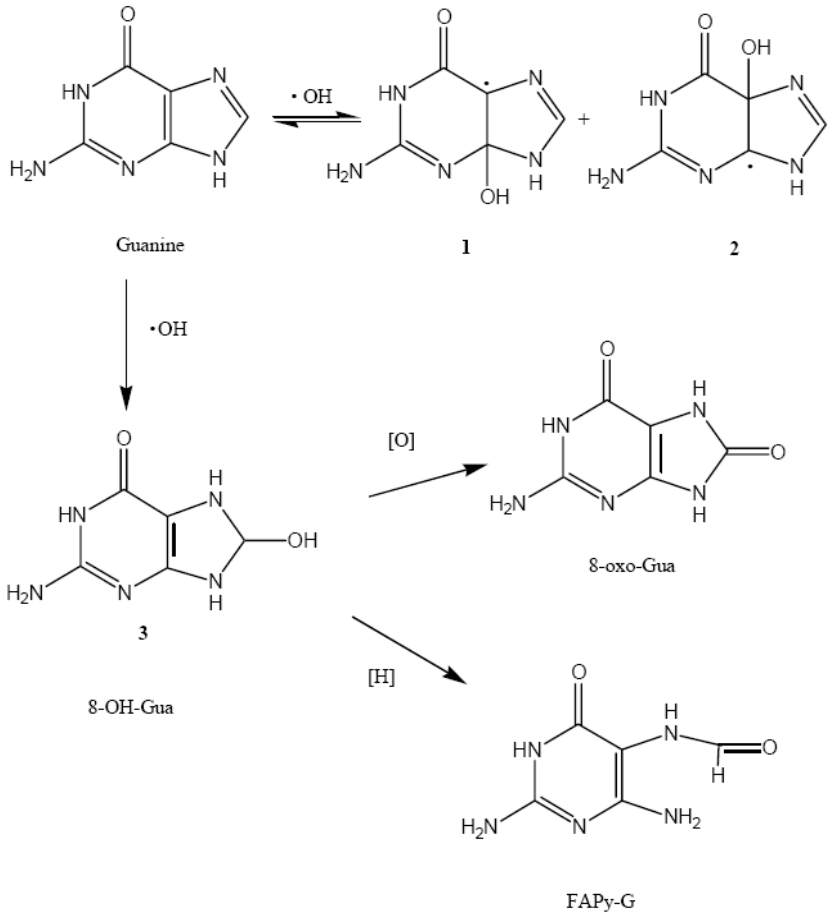

Figure 1.5 Schematic representation for the reaction of guanine with hydroxyl radicals

1.7.2 Carbon centered radicals

The study of DNA damage by carbon-centered radicals began with the discovery of enediyne antibiotics. There was EPR studies that the carbon radicals generated from dihydropyrazines are effective in causing DNA cleavage (Kashige *et al*, 2000). There are two ways carbon centered radicals can be formed *in vivo*. One is under conditions of high oxidative stress and the other is by abnormal metabolism of carbohydrates and amino acids brought about by genetic disorders.

It is thought that alkyl radicals are able to abstract hydrogen atoms from the sugar moiety in nucleotides leading to alkylation and consequently DNA damage. Carbon radicals induced DNA damage by attacking guanine and adenine bases of DNA forming 8-alkylpurines which can ultimately lead to DNA damage (Figure 1.6) (Maeda *et al*, 1974).

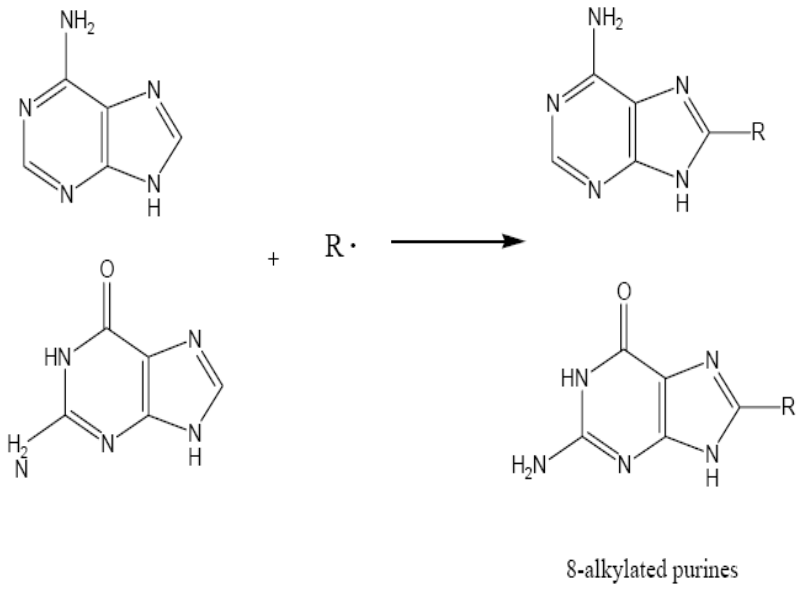

Figure 1.6 Schematic representation of carbon centered metabolites where R represents a carbon radical

1.8 Fenton-Menton reaction

Metals have special oxygen transfer properties which improve the use of hydrogen peroxide. Actually, metals have a strong catalytic power to generate highly reactive hydroxyl radicals (·OH). Below are the mechanism of Fenton-Menton reaction where M represents metal.

$$M^{2+} + H_2O_2 \rightarrow M^{+} + 2H^{+} + O_2^{\cdot -}$$
$$M^{+} + H_2O_2 \rightarrow M^{2+} + \cdot OH + OH^{-}$$
$$M^{+} + O_2 \rightarrow M^{2+} + O_2^{\cdot -}$$
$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + 2 \cdot OH$$
$$O_2^{\cdot -} + M^{2+} \rightarrow {}^1O_2 + M^{+}$$

Figure 1.7 Mechanism of Fenton-Menton reaction (M represents metal)

1.9 MTT assay

MTT a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan, giving a purple color. This mostly happens in mitochondria, and as such it is in a measure of mitochondrial activity. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials.

Yellow MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product. The absorbance of this colored solution can be quantified by measuring absorbance at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed.

This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells.

However, it is important to keep in mind that other viability tests sometimes give completely different results, as many different conditions can increase or decrease metabolic activity. Changes in metabolic activity can give large changes in MTT results although the number of viable cells maybe constant. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve.

1.10 IC_{50}

IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. Often, the compound in question is a drug candidate. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC_{50}). It is commonly used as a measure of antagonist drug potency in pharmacological research. Sometimes, it is also converted to the pIC_{50} scale (-log IC_{50}), in which higher values indicate exponentially greater potency. According to the Food and Drug Administration, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. It is comparable to an EC_{50} for agonist drugs. EC_{50} also represents the plasma concentration required for obtaining 50% of a maximum effect *in vivo* (Xu *et al*.,2004).

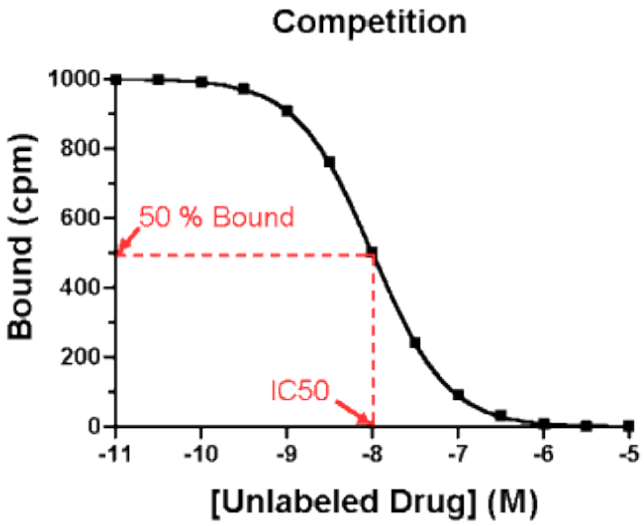

Figure 1.8 The graph of IC_{50}

1.11 Objectives and scope of study

The objective of this research is to synthesize and characterize a series of complexes formed by the reaction of cobalt(II)chloride hexahydrate, $CoCl_2 \cdot 6H_2O$ or zinc(II)acetate dihydrate, $Zn(CH_3COO)_2 \cdot 2H_2O$ with carboxylic acids.

1. To study the interaction of metal complexes with DNA
2. To study the DNA cleavage ability and the cyctotoxic activity of metal complexes
3. To investigate the mechanism of the interaction between the metal complexes and DNA

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

Table 2.1 illustrates the melting points, purify, molecular weight and the suppliers for every chemical that used in the research. The chemicals were used without further purification.

Table 2.1 Chemicals used in the research

Name	Features			Supplier
	Melting Point (°C)	Purity (%)	Mr	
Cobalt(II) chloride hexahydrate	86°C	≥98.0%	237.93	Hamburg Chemical GmbH
Zinc(II) acetate dihydrate	100°C	≥97.5%	219.5	Fluka Chemika
2-Picolinic acid	135-136°C	98%	123.11	Fluka Chemika
Pyrazine-2-carboxylic acid	-21-22°C	99%	107.11	Aldrich
Malonic acid	132-135°C	≥98%	104.06	Fluka Chemika
Maleic acid	137-140°C	≥99%	116.08	Fluka Chemika
Glycine	<30°C	100%	75.07	Fisher Chemika
4-Aminobenzoic acid	187-189°C	99.5%	137.14	G.P.R.
Glycolic acid	75°C	97%	76.05	Fluka Chemika
Pyrazine-2,5-dicarboxylic acid dihydrate	190°C	≤97%	204.14	Fluka Chemika
Quinolinic acid	185°C	~99%	167.12	Fluka Chemika
3,5-Dinitrobenzoic acid	205°C	99.5%	212.12	BDH Chemicals

2.2 Experimental

Cobalt(II)chloride hexahydrate or zinc(II)acetate dihydrate was used as the starting material to prepare these complexes. Cobalt(II)chloride hexahydrate (1.00 g, 0.0042 mol) was dissolved in 20 mL of distilled water. The red solution was put into a two-neck round flask. Reagents in ratio of 1:2, starting material:ligand were used and refluxs for 3 hours.

Zinc(II)acetate dihydrate (1.00 g, 0.0046 mol) was dissolved in 20 mL of distilled water. The colourless solution was put into a two-neck round flask. Reagents in ratio of 1:2, starting material:ligand were used and refluxs for 3 hours.

All the ligands were dissolved in 30 mL of distilled water before mixed into the two-neck round flask.

2.3 Methods of characterization

2.3.1 Melting points

A Gallenkamp Variable Heater in a capillary tube in was used to determine the melting point of each complex. The complex was observed through a window of the heater and the temperature was recorded when the complex started to melt.

2.3.2 Conductivity measurement

The measurement was conducted on 10^{-3} M of the complex solution by using the CyberScan 500 instrument.

2.3.3 Fourier transform infrared spectroscopy (FT-IR) analysis

The IR spectrum of the each complex was taken on a Perkin Elmer FTIR System 2000 spectrophotometer in the frequency range 4000–400 cm^{-1} in the room temperature. Every dry analyte was ground with sodium bromide (KBr) in the ratio of 1:10 to obtain fine and evenly ground powder. The powder was used to form pellet using specific mould and pressed under 7 tonne by hydrolic press under vaccum.

2.3.4 Microanalysis CHN

A CHNS/O Analyzer (Perkin-Elmer Series II 2400) was used to determine the percentage of elements carbon, hydrogen and nitrogen.

2.3.5 Atomic absorption spectrometry (AAS) analysis

This analysis was carried out on a Perkin-Elmer Atomic Absorption Spectroscopy Model 310 to determine the quantitative value of cobalt and zinc in each complex. About 0.02 g of each complexes was dissolved in 10 mL of

concentrated nitric acid and diluted with 2% nitric acid until 50 mL in a volumetric flask.

2.3.6 Ultraviolet-visible spectrometry (UV-Vis) analysis

About 0.02 g of each complexes was dissolved in distilled water. The UV-Visible spectra were measured on a Perkin-Elmer Lambda 35 in the range of 400-4000 nm. Dilution of each complexes was done until a nice peak obtained.

2.3.7 Cyclic voltammetry (CV) analysis

This analysis was done by using BAS Epsilon. The measurements were made around 10^{-3} M of the sample tested in distilled water using 0.1 M potassium chloride (KCl) as supporting electrolyte. A three-electrod (working, reference and counter) configuration cell was used in this analysis. A platinum electrode was used as counter electrode and all potentials were measured as the Ag/AgCl reference electrode. The sample solution was purged with nitrogen gas for 15 minutes to remove the oxygen from the solution and an atmosphere of nitrogen (N_2) was maintained throughout eight cyclic voltammetric scans with the scan rate of 50 mV/s.

2.3.8 Thermogravimetric (TGA) analysis

Thermogravimetric analysis of each complex was measured with a Mettler Toledo TGA/SDTA851e Thermal Gravimetric Analyzer. About 0.001-0.002 g of

each sample was placed in a platinum crucible and transferred to the heating furnace of the analyzer unit. The analysis was performed under a dynamic inert atmosphere (N_2) with a flow rate of 30 mL min^{-1}. All samples were heated over the temperature range 30-900 ºC with heating rate of 20 ºC min^{-1}. All TGA data acquisitions and data processing were done using Mettler Toledo STARe version 8.10 software. The results were presented as differential thermal gravimetric curves.

2.3.9 X-ray crystallography

Complexes that were obtained as single crystals were determine on single crystal X-ray diffractometer equipped-monochromated Mo-Kα radiation (λ=0.71073 Å) in order to reveal the crystal structure of the complexes. The intensity data for the titled complex was collected at 100.0 K using Bruker SMART APEX2 CCD or at 273.0 K using a Bruker SMART APEX CCD. The Oxford Cyrosystem Cobra low-temperature attachment to SMART APEX2 CCD was also used to collect the low-temperature data. In addition, the data collected were refined using the program SAINT and a semi-empirical absorption correction was applied to the data using SADABS. The structure was solved by direct method and refined against F^2 by full – matrix least squares using SHELXTL. Crystal data and experimental details of the structure determinations are listed in supplemental material.

2.3.10 DNA cleavage experiments

Agarose gel electrophoresis experiments were carried out on supercoiled plasmid pBR322 (4.4 kb). All samples in TAE buffer [40 mM Tris, 20 mM EDTA; pH 8.3; Tris=tris(hydroxymethyl)aminomethane; EDTA=disodium salt of ethylene-diaminetetra-acetic acid] were incubated at 37 °C for 2 hours and then electrophoresed on 1 % agarose gel for 3 hours at 50 V. 0.0125 µg/µL DNA with the increasing concentration for each sample were incubated in the presence of a specified concentration of hydrogen peroxide (H_2O_2). At the end of electrophoresis, the gel was stained with ethidium bromide (EtBr) before being photographed under UV light using a Syngene Bio Imaging System.

In addition, the effect of hydroxyl radical scavengers (sodium azide, t-butanol, D-mannitol and DMSO) and hydrogen peroxide scavenger (KI) on the nucleolytic activity of these complexes were investigated. For each experiment, a 20 µL mixture consisting of the appropriate volumes of stock solution of DNA, complex dissolved in distilled water, additional buffer, scavengers and aqueous H_2O_2 (last component added) was incubated at 37 °C for 2 hours before electrophoresis.

2.3.11 MTT assay

The human liver cancer cell line, HepG2 used in this study was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Co., Carlsbad, California, U.S.A) supplemented with 10 % heat-inactivated fetal calf serum (Invitrogen Co., Carlsbad, California, U.S.A), 100 µg/mL penicilin and 100 µg/mL

streptomycin (Flowlab, Sydney, Australia). Cells were kept in the logarithmic growth phase by routine passage every 2 or 3 days using 0.025 % trypsin-EDTA treatment.

MTT assay was used to test the cytotoxicity of sample complexes against human liver cells. MTT assay was performed in 96-flat bottom well plates. 100 µL of cell (1×10^5 cell/mL) was seeded into each well except the outer surrounding wells (blank) of the plate and incubated for 24 hours. Then, 100 µL of the dilution series of complex was added into each well and the plate was incubated for another 72 hours. The MTT solution was prepared at a concentration of 5 mg/mL in phosphate buffered saline (PBS). Later, 20 µL of MTT was then added to each well and the plate was incubated for another 4 hours. The medium in the wells of the plate were removed and 200 µL of DMSO was added to dissolve the formazan crystal (Xu *et al*.,2004). The absorbance of each well was measured in a microplate reader at 570 nm with a reference wavelength of 630 nm. The percentage of cell visibility was calculated with the formula:

$$\frac{Average\ A_{570}\ value\ for\ live\ cell\ (test)}{Average\ A_{570}\ value\ for\ live\ cell\ (control)} \times 100\%$$

The IC_{50} value was obtained from the graphs of the complex was drawn.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Characterization of diaquabis(2-pyrazinecarboxylato)cobalt(II) and diaquabis(2-pyrazinecarboxylato)zinc(II)

The complexes diaquabis(2-pyrazinecarboxylato)cobalt(II) and diaquabis(2-pyrazinecarboxylato)zinc(II) formed by the reaction of $CoCl_2 \cdot 6H_2O$ and $Zn(CH_3COO)_2 \cdot 2H_2O$ with pyrazine-2-carboxylic acid are represented by Co(p-2c) and Zn(p-2c) respectively.

3.1.1 Determination of physical properties

Orange crystals of Co(p-2c) and white crystals of Zn(p-2c) were obtained. Both these crystals are neutral by referring to the molar conductance table and soluble in polar solvents (Table 3.1.2). Co(p-2c) and Zn(p-2c) decomposed before reaching their melting points at 312.4-312.5 °C and 328.6-328.7 °C respectively (Table 3.1.1).

Table 3.1.1 The physical properties of Co(p-2c) and Zn(p-2c)

Complex	Co(p-2c)	Zn(p-2c)
Molecular Formula	$Co(C_5H_3O_2N_2)_2(H_2O)_2$	$Zn(C_5H_3O_2N_2)_2(H_2O)_2$
Molar Mass	341.15 g/mole	347.60 g/mole
Theoretical Value	1.43 g	1.60 g
Experimental Value	1.34 g	1.12 g
Yield	93%	70%
Appearance	Orange crystals	White crystals
Melting Points	312.4-312.5°C (Decompose)	328.6-328.7°C (Decompose)
Solubility	Dissolve in all polar solvents	Dissolve in all polar solvents
Conductivity	79 $cm^2ohm^{-1}mol^{-1}$	17 $cm^2ohm^{-1}mol^{-1}$

Table 3.1.2 Molar conductance, Λ of electrolyte solution at 25°C

Number of ion	Molar conductance, Λ
2	118-131
3	235-273
4	408-435
5	~560

3.1.2 Fourier transform infrared spectroscopy (FT-IR) analysis

For the correlation of the infrared spectra with the structure of metal carboxylates the difference between the asymmetric and symmetric carboxylate stretches [$\Delta=\nu_{as}(COO^-)-\nu_s(COO^-)$] is often used. This value is approximately 170 cm^{-1} for ionic carboxylate group, indicated the group which does not interact strongly with metal ion (Deacon and Philip, 1980). In monodentate coordination the redistribution of electron density takes place, which shift the asymmetric carboxylate stretch, $\nu_{as}(COO^-)$, to higher wavenumbers in comparison to the ionic group. Consequently the Δ value for monodentate carboxylate coordination is higher. While, bidentate coordination shifts the position of the asymmetric carboxylate stretch