SYNTHESIS AND BIOCHEMICAL CHARACTERIZATION OF NOVEL METALLO-SALPHEN DERIVATIVES

by

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Abstract

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In an effort to find a novel metal complex that can be used in cancer therapy a series of Fe(III)-salphen derivatives were synthesized and then characterized biochemically. Cytotoxicity assay revealed that Fe(III)-salphen derivatives are toxic to human breast cancer (MCF7) and human colon cancer (CCL228) cells at very low concentrations with IC_{50} values in the range of 0.31 to 1.48 μM, which is much lower than that of Cisplatin (IC_{50} 18 ± 2 μM). Caspase-3/7 assay and nuclear staining revealed that these compounds kill the cells by inducing apoptosis as signified by the condensation and fragmentation of the nucleus and the increased activity of caspase-3/7. Ascorbic acid assay revealed that the Fe(III)-salphen derivative (compound 2, CH_3) induces apoptosis by oxidative stress as the viability of compound 2 treated cells was significantly increased by co-treating with ascorbic acid. As cell cycle regulatory genes are overexpressed in tumor cells the expression of cyclins (A, B, D, and E) was evaluated at mRNA levels after treatment of CCL228 cells with compound 2 (CH_3) and Cisplatin. The results showed that the toxicity of these compounds is not related to the overexpression of these genes although some cyclins seem to be up-regulated in Cisplatin treated cells.

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Chapter 1

Introduction

1.1 Metal Complexes in Medicine

Metal ions are of great importance to the living system as they play an important role in enzymatic activity because they are present in the active site of enzymes. Metals such as zinc, iron, copper, cadmium, nickel, magnesium, and chromium are required for normal biological functioning. Deficiency of these metals is associated with diseases such as iron deficiency anemia, growth retardation, and brain disease. Although these metals are required for proper functioning, exceeding the normal amounts can cause heavy metal toxicity. Despite their known toxic effects, metals and metal complexes have been successfully exploited for their medicinal usage for thousands of years (*1*). For example, antimony, bismuth, gold, iron, silver, and platinum have been used as anti-protozoal, anti-ulcer, anti-inflammatory, anti-arthritic, anti-malarial, anti-microbial, and anti-cancer agents respectively (*1*). Severe cases of rheumatoid arthritis are clinically treated by injection of transition gold complexes; severe burns are treated with silver sulfazine to prevent them from bacterial infections; and proliferation of inflammatory brain cells is controlled by metallic gold treatment (*2*). The toxicity of metals is well exploited in cancer therapy by modifying them to selectively target tumor cells. Metals or metal-containing compounds have been investigated for the treatment of cancer and leukemia as early as the sixteenth century (*1*). Since then a wide range of compounds containing transition metals and main group elements has been studied for their anticancer properties (*1*).

Transition metal complexes are valuable in medicinal chemistry because they include metals that are naturally present at the active sites of enzymes. One of the major

factors that determine the biological activity of metal complexes is the oxidation state of the of central metal ion (*3*). Ligands are also important players in drug design that allow scientists to modify the oral/systemic bioavailability of the metal ions and to target specific tissues or enzymes (*3*). Transition metal based compounds are well studied in drug research because they exhibit multiple oxidation states and can form stable complexes by coordinating with various ligands (*3*). Transition metal complexes work by crosslinking the DNA of cancerous cells which leads to cell death (*2*). They are also known to affect the cellular function by inhibiting enzymatic activities. Their coordination chemistry allows them to displace the native metals that are present in active sites of enzymes and inhibit enzyme functions (*4*). Transition metal complexes have high affinity to DNA and proteins because of the presence of a positively charged central metal ion that allows them to interact with the negatively charged phosphate backbone of DNA and to the negatively charged carboxyl and sulfhydryl groups of proteins. Transition metal complexes are also known to induce oxidative stress by generating free radicals and reactive oxygen species (ROS; O^{2-}, •OH, NO•, and H_2O_2) that are detrimental to normal and cancer cells as they can cause oxidative damage to the DNA (*5*). The medicinal importance of metal complexes is undeniable, however many heavy metals are also associated with several toxicity issues. So the study on reducing the side effects of currently available metal based drugs is an emerging field of research.

1.2 Metal Complexes as Anticancer Agents

The success of Cisplatin as an anticancer drug brought about a major breakthrough in the use of transition metals in medicinal chemistry and anticancer research. The heavy metals that were previously associated with lethal diseases are now being used to treat them. Cisplatin (cis-$[Pt(NH_3)_2Cl_2]$) was the first platinum based

complex that was used to treat cancer. It was synthesized in 1844 and was known as Peyrone's chloride (1). Its discovery as an inhibitor of *E. coli* cell division was unplanned and was found while studying the effect of electric field on bacterial growth using platinum electrodes (*6*). It was first reported by Rosenberg in 1965 (*7*) and was successfully used to treat testicular cancer in 1978 (*6*). Today, Cisplatin or other platinum based drugs are almost always used in combination with other chemotherapeutic drugs (*6*). The success of Cisplatin arises due to its efficient anticancer properties and its unique toxicity profile that allows it to be used in combination with other drugs (1). After the success of Cisplatin, over three thousand platinum based complexes have been synthesized and studied for their anticancer properties. Among them Cisplatin, Carboplatin, Oxaliplatin and Nedaplatin (shown in figure 1-1) are the only ones used clinically and some of them are still under clinical investigation (1).

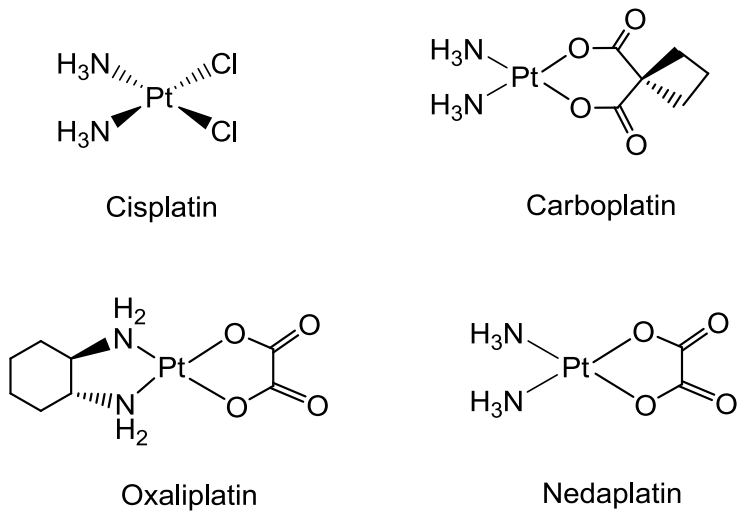
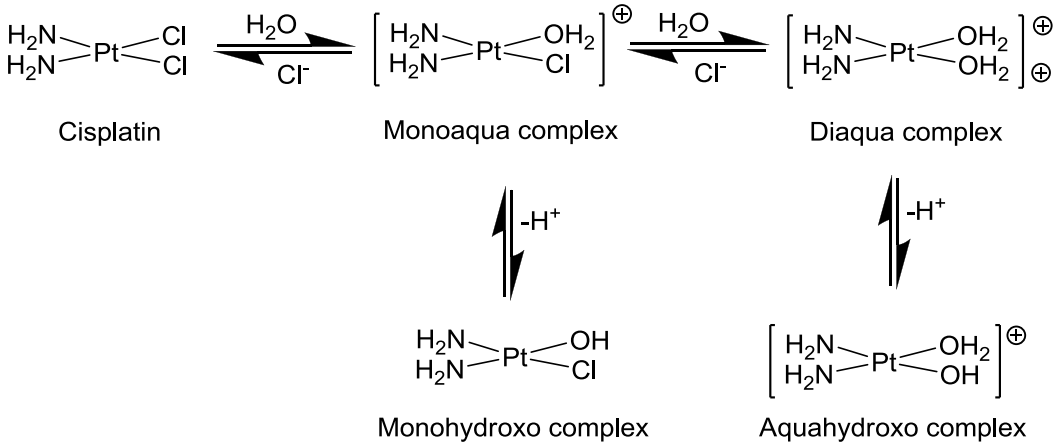


Figure 1-1 Structures of clinically used platinum based anticancer drugs redrawn from reference (*8*)



Scheme 1-1 Hydrolysis of Cisplatin to form biologically active platinum complex redrawn from reference (*9*)

Cisplatin enters the cells by passive diffusion and undergoes hydrolysis due to the low chloride concentration (20 µM) inside the cells to form the activated diaqua species (scheme 1-1) that can irreversibly bind the two adjacent guanine bases (most electron rich site) and crosslink the DNA of the target cells (*10*). The cross-linked DNA cannot replicate and the cell is forced to undergo apoptosis. Although, Cisplatin is the most effective drug that is available to treat cancer, it has several limitations. The therapeutic effects of Cisplatin bring along multiple side effects that arise due to its non-specificity which can kill the cells and harm non-target tissues (*6*). Cisplatin is also prone to resistance phenomenon as cancer cells can develop insensitivity towards Cisplatin due to decreased cellular uptake, increased DNA repair, and decreased capability to crosslink DNA (*11*). Another drawback of Cisplatin is that it cannot be used to target all types of cancer because it does not show toxic response in them. The use of platinum based complexes as anticancer drugs is limited mainly due to its nephrotoxicity (*12*). For example, Cisplatin generates increased levels of reactive oxygen species (ROS) that kill the tubular cells of the kidney and make it inefficient to remove toxic substances from body causing nephrotoxicity (*12*). Several studies have been conducted to control

Cisplatin toxicity so that it can be safely administered to patients. For example, Kaeidi et. al., have shown that Cisplatin toxicity in human tubular epithelial cells can be reduced by exposing the cells to hyperoxic condition. They have proposed that initiating mild oxidative stress before treatment with Cisplatin helps the cells to enhance their defense mechanisms to scavenge for reactive oxygen species generated by Cisplatin (*12*). Due to the limitations of the currently available anticancer drugs there is always an increasing demand to develop novel drugs that have more specificity towards cancerous cells, fewer side effects, and that can target a broad range of tumors (*11*).

1.2.1 Non-Platinum Based Compounds as Potential Anticancer Agents

The limitations of platinum based anticancer drugs towards cancer therapy have encouraged scientists to expand their research on non-platinum metal complexes with a hope of finding a drug with less severe side effects. Researchers have found some success in drug design by varying the central metal ion and the type of ligand used. For example, iron, cobalt, and gold based metal complexes have been evaluated successfully in preclinical studies; and titanium, ruthenium, and gallium based complexes are being evaluated in phase I and phase II clinical trials *(8)*.

Besides platinum several other transition metals are being exploited in drug development because of their wide range of coordination numbers, oxidation states, and ligand binding affinities (*13*). Ferrocenium complexes are the first iron based compounds that were reported to have anticancer properties (14). Substituted ferrocenes (figure 1-2) are an important class of iron based compounds that mediate toxic effects in the cells by inducing oxidative stress. In the tumor cells, ferrocene (Fe^{2+}) can be oxidized to ferrocenium ions (Fe^{3+}) that can damage DNA by generating reactive oxygen species (ROS) (*8*). Similarly, iron carbonyl nucleosides and Fe(II) complexes with pentadentate pyridyl ligands (figure 1-2) also exhibit anti-proliferative properties (*8*).

Cobalt is an essential trace element present in the active site of co-enzymes (i.e. cobalamins). Complexes based on cobalt have been reported to display anti-microbial and anti-tumor properties (*15*). Cobalt complexes when coordinated to certain anti-inflammatory, anti-bacterial, and anti-fungal drugs can enhance or modify their medicinal properties. For example, coordination of $Co_2(CO)_6$ to aspirin (non-steroidal anti-inflammatory drug) via an alkyne bond (Co-ASS, figure 1-2) improves its lipophilicity and bioactivity (*13*). Co-ASS was shown to exhibit anti-proliferative properties in murine leukemia cells most likely due to increased cyclooxygenase inhibition (*8*). Lipophilic Co-ASS can accumulate at high levels inside cells than its aspirin counterpart and enhance the inhibition of cyclooxygenase (*13*), (*15*). Cobalt(III) complexes are also known to act as a hypoxia selective anticancer agent as they can be reduced to more active cobalt(II) in hypoxic condition in the tumor regions (*8*).

Gold is a valuable metal that was used in ancient Chinese and Arabic medicine. The medicinal properties of gold became of scientific interest after the discovery of anti-bacterial properties of $Au(CN)^{2-}$ and the use of gold thiolate for the treatment of rheumatoid arthritis (*16*). Due to the ability of Au(II) to form square planar geometry like Pt(II) researchers began to evaluate the anti-proliferative activity of gold compounds. Auranofin (figure 1-2) was the first gold complex that was shown to inhibit the growth of human cervical cancer (*HeLa*) cells (*16*). Similarly, other analogues of auranofin were also shown to have cytotoxic effects that were driven by inhibition of mitichondrial thiredoxin reductase (*16*). Thioredoxin reductase is a selenoenzyme that can reduce the antioxidant protein thioredoxin (*8*). Gold(I) and gold(III) complexes might be involved in the ligand exchange process with selenocysteine and cysteine residues in the active site of thioredoxin reductase thereby inhibiting the enzyme (*8*). Likewise, the inhibitory

properties of a gold phosphol complex (figure 1-2) on human glioblastoma cells was also due to the inhibition of glutathione reductase and thioredoxin reductase (*8*).

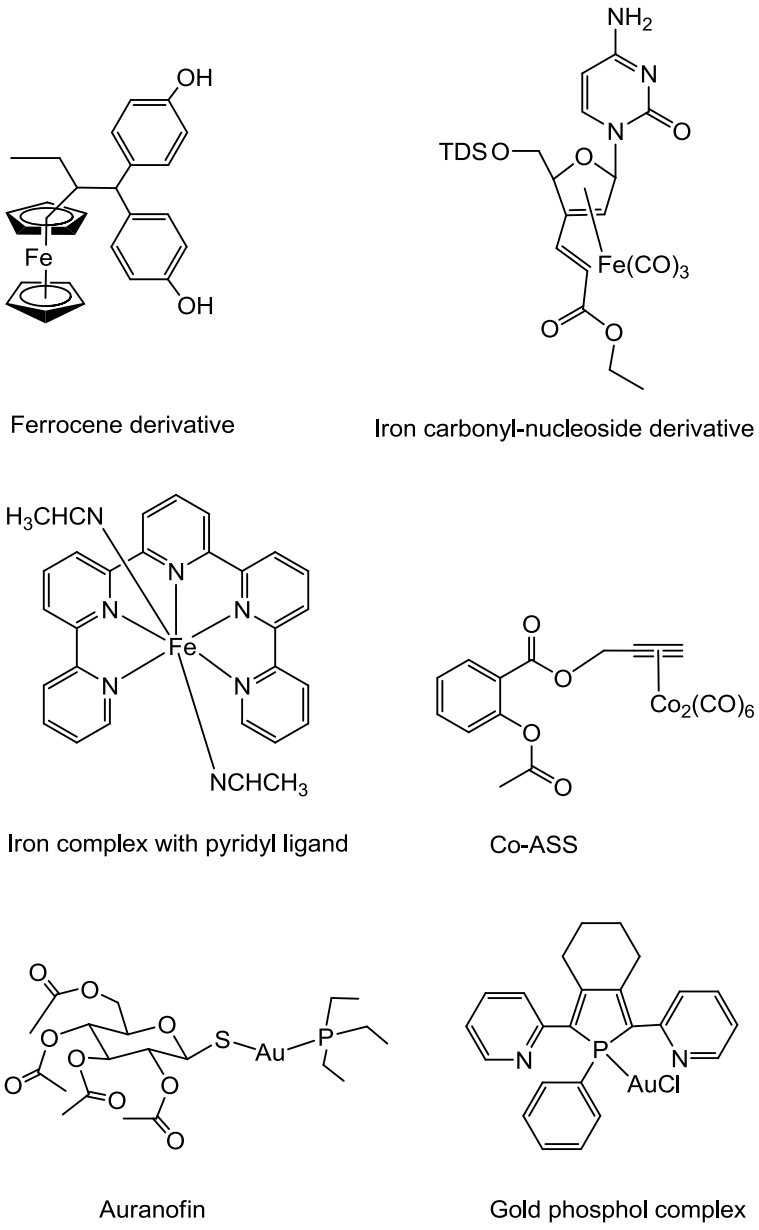


Figure 1-2 Structures of potential non-platinum anticancer agents redrawn from reference (*8*) and (*16*)

1.2.2 Non-Platinum Based Anticancer Drugs in Clinical Trials

Several metal base compounds have shown promising results in pre-clinical studies but only a few of them have been evaluated in clinical trials. Titanium and gallium based complexes have shown promising results in phase I clinical trials but were unsuccessful in phase II trials. Ruthenium complexes have been successfully evaluated in phase II trials and have fewer side effects than Cisplatin *(8).*

Titanocene dichloride (figure 1-3) was the first metallocene that was shown to have anticancer properties (*1*). It was shown to induce apoptosis by covalently binding to the phosphate backbone of DNA and inhibiting DNA synthesis (*8*). It showed promising results in phase I clinical trials, but due to its low solubility in aqueous medium and fast hydrolysis under physiological conditions, it failed in phase II trials (*8*). Budotitane (figure 1-3) and its derivatives are another class of titanium based anticancer drugs that were shown to be active in pre-clinical studies but failed to show any response in clinical studies (*8*). Titanium salan complexes (figure 1-3) are the third class of titanium based anticancer drugs that are more stable in aqueous medium than titanocene and budotitane and have displayed promising results in vivo and in vitro cytotoxicity assay (*17*).

Gallium is most commonly found as Ga(III) and it closely resembles Fe(III) in terms of its electrical charge, ion diameter, coordination number, and electron configuration (*1*), due to which it is transported by transferrin and is found to be accumulated inside lysozymes in the cells (*8*). Gallium nitrate, gallium chloride, and gallium maltolate have been investigated in clinical trials. Among them gallium nitrate has been proved to be active against lymphoma and bladder cancer (*18*). Gallium toxicity is enhanced by transferrin receptors that increase its cellular uptake; once inside the cells, gallium can inhibit DNA synthesis by targeting ribonucleotide reductase (*8*), (*1*). Gallium is also known to mediate its toxicity by disrupting iron transport and by blocking the cell

cycle in the S-phase (*18*). Gallium nitrate was not successful in phase II clinical trials because it failed to show any response in melanoma and breast cancer; and was associated with toxic side effects when used in combination therapy against metastatic urothelial carcinomas (*8*). KP46 (FFCC11), [tris(8-quinolinolato)gallium(III)], is a lipophilic gallium complex that is currently undergoing phase I clinical trials (figure 1-3). KP46 is hydrolytically stable and has better bioavailability after oral administration due to which it exhibits increased cytotoxicity and antitumor activity (*8*).

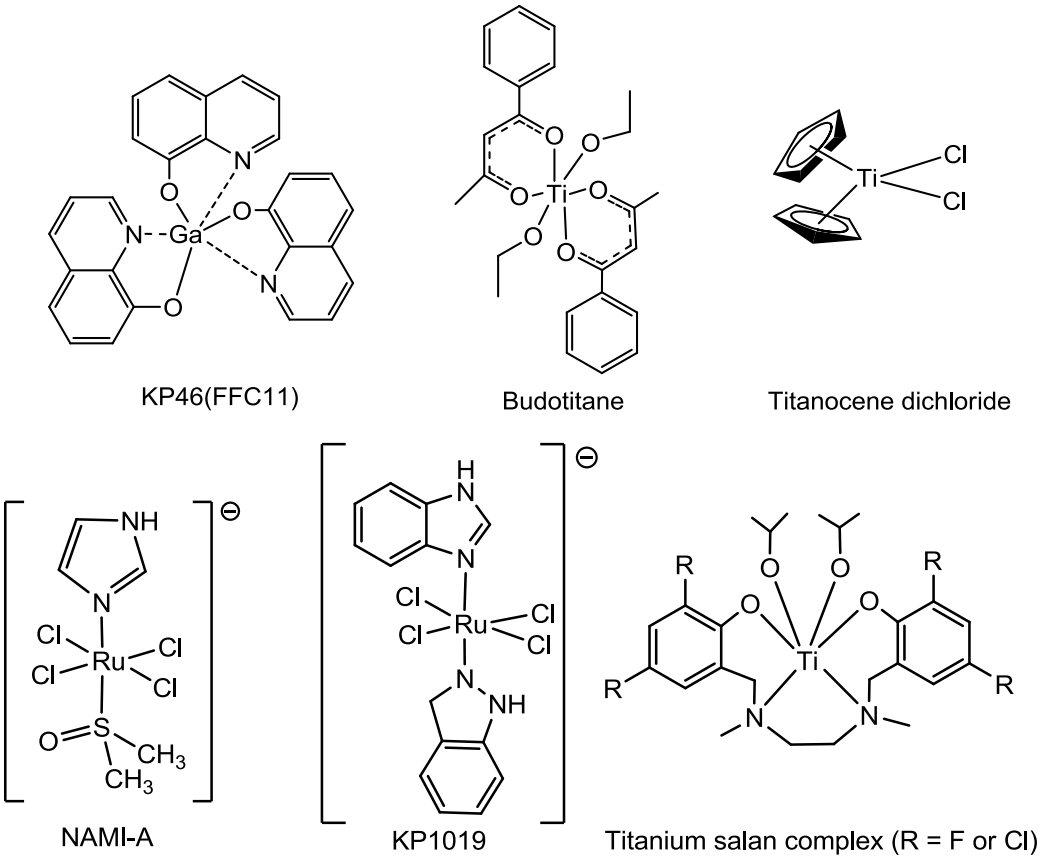


Figure 1-3 Structures of non-platinum metal complexes
redrawn from reference (*8*) and (*17*)

Several ruthenium based drugs have been synthesized and investigated for their cytotoxicity because of its ability to exist in multiple oxidation states (*19*). Among them

KP1019 (trans-[tetrachlorobis(1H-indazole)ruthenate(III)]) and NAMI-A (imidazolium-trans-DMSO-imidazole-tetrachlororuthenate) have successfully completed phase I clinical trials and have fewer and less severe side effects towards platinum resistant tumors (*20*). The biological activity of ruthenium based drugs is dependent on their oxidation state (*19*). The advantages of ruthenium complexes over platinum complexes are that they accumulate preferentially in neoplastic tissues over normal tissue, they provide selective toxicity, and are more efficient towards hypoxic tumors that are resistant to chemotherapy (*20*). Hypoxic environment and lower pH is required to reduce inactive ruthenium(III) to active ruthenium(II) which is only available at the tumor sites and this helps in lowering ruthenium toxicity (*20*). Iron like behavior of ruthenium allows it to bind to serum transferrin and albumin. Serum transferrin and album solubilize and transport iron and help to reduce iron toxicity (*19*). Rapidly dividing cells have a higher demand for iron thereby increasing the number of transferrin receptors on their cell surfaces. Transferrin receptors bind to ruthenium, enhance its accumulation in cancer cells, lower the toxicity in non-cancer cells, and increase the selectivity of ruthenium based drugs towards cancer cells (*19*). The drawback of this class of drugs is its unknown mode of action. More research has yet to be done to elicit the mode of action for ruthenium based drugs so that they can be designed to specifically target different types of cancer (*19*).

1.3 Anticancer Drugs Induce Apoptosis

Apoptosis is a programmed cell death that occurs in multicellular organisms to maintain a balance between cell proliferation and cell death (*21*). Apoptosis is a natural process that can be initiated or inhibited by different physiological and pathological stimuli. Apoptosis leads to the separation of the plasma membrane from the cytoplasm, bulging of the membrane, reduction of cytoplasmic volume, condensation and

fragmentation of chromatin DNA, formation of apoptotic bodies, and engulfment of the cell remains by the neighboring cells (*22*)-(*23*). Unlike necrosis, apoptosis occurs without damaging the neighboring cells and is not associated with inflammatory response (*24*). Apoptosis maintains the homeostasis in healthy adult tissues, eliminates unwanted cells during normal embryonic development, occurs spontaneously in untreated cancers, and is involved in the treatment of some types of tumors (*24*). Apoptosis can be induced in live cells by redox active agents that can elevate the oxidative stress or damage DNA (*21*). Decreased apoptosis may lead to several autoimmune disorders and cancer whereas increased apoptosis may lead to neurodegenerative diseases and stroke (*22*). Apoptotic pathway is defective in almost all tumor cells which increases the resistance of tumor cells to chemotherapy (*25*). Hence, by targeting the apoptotic pathway in tumor cells we can overcome the tumor resistance to chemotherapy.

Apoptosis is initiated by a class of cysteine-aspartic proteases called caspases which remain dormant until they receive an apoptotic signal (*26*). Hormones, cytokines, viruses, anticancer agents, etc. can trigger the activation of caspases via the death receptor pathway (extrinsic) or via the mitochondrial pathway (intrinsic) (*26*). Anticancer drugs are known to induce apoptosis mainly via the intrinsic or mitochondrial pathway (*27*). In the extrinsic pathway, death-inducing signaling complex (DISC) is formed when death ligands (TNFα, FasL, etc.) bind to their receptors (TNFR1, FasR) where the initiator procaspase-8 is recruited by FADD (FAS-associated death domain protein) and is activated by autocatalytic cleavage (figure 1-5) (*25*). In the intrinsic pathway, damage to the cellular DNA by viral infections, toxins, free radicals, and radiation induces the release of pro-apoptotic proteins into the cytosol due to change in the inner mitochondrial membrane and loss of transmembrane potential (*28*). Pro-apoptotic events in the mitochondria are regulated by the Bcl-2 family of proteins and the tumor suppressor

protein p53 (*28*). p53 is a transcription factor which is expressed at low levels in healthy cells and is overexpressed in cancer cells where it functions to get rid of the cancerous cells by inducing apoptosis (*29*). p53 can be activated by several intrinsic and extrinsic factors and p53 is known to induce apoptosis mainly via mitochondrial pathway (*29*). The basic role of p53 in apoptosis is to activate transcription of pro-apoptotic genes (like genes that encode Bcl-2 family of proteins) so that the body can balance the cell proliferation with death (*28*)-(*29*).

Bcl-2 family of proteins is an important regulator of apoptosis. The Bcl-2 family of pro-apoptotic proteins (Bcl-10, BAX, BID, BAD, BIK, Blk and BIM) activate mitochondrial pathway and induce the release of cytochrome c into the cytosol (*25*). Cytochrome c binds to the apoptotic protease activating factor 1 (APAF1) to form the apoptosome and activates the initiator caspase-9 (*25*). Activated initiator caspases cleave and activate executioner caspases (caspase-3, caspase-6, and caspase-7) which then initiate the enzymatic cascade that cleave the cellular death substrates and lead to the biochemical and morphological changes associated with apoptosis (*25*). The Bcl-2 family of anti-apoptotic proteins (Bcl-2, Bcl-x, Bcl-xL, Bcl-XS, Bcl-w, and BAG) are known to block apoptosis (*28*). Anticancer research has recently targeted the upregulation of pro-apoptotic proteins or suppression of anti-apoptotic proteins as a potential therapy for cancer (*28*). Figure 1-4, shows the two different pathways for apoptosis and BID as the mediator between the two pathways (*22*).

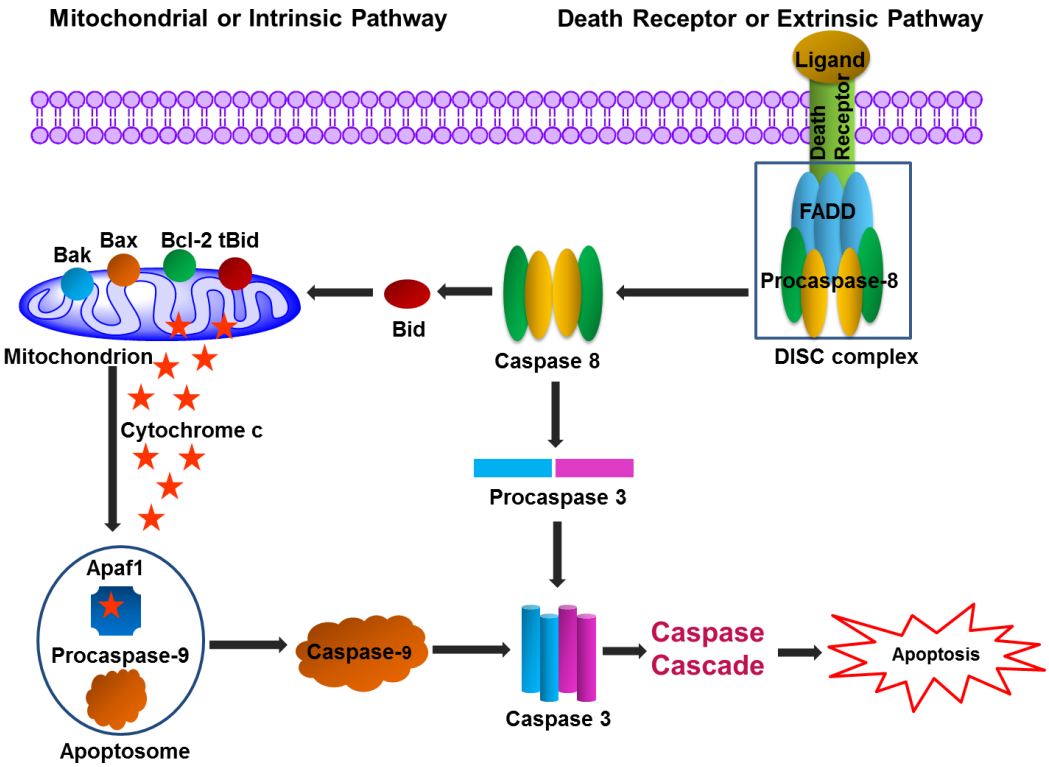
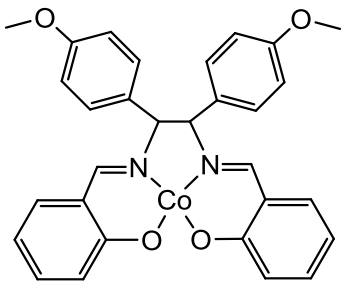


Figure 1-4 Pathway of apoptosis
redrawn from reference (*30*)

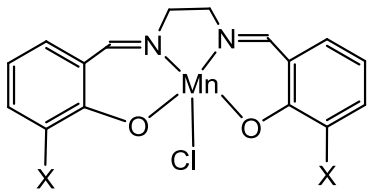
1.4 Biological Significance of Metallo-Salen Complexes

The ability of Schiff bases to form complexes with a wide range of transition metal ions make them “privileged ligands” (*31*). Metal complexes of Schiff bases demonstrate antiviral, antifungal, antimicrobial, plant growth regulator, insecticidal, and anticancer properties which make them an important class of compounds (*32*). Several transition metal Schiff base complexes are being developed and studied for their potential anti-tumor activities. Among them metal complexes of salen (N,N′-bis(salicylidene)-1,2-ethylenediamine) derivatives have been a recent target for anticancer drug research (*33*). Metallo-salen complexes were first developed as catalyst for the oxidation of olefins and

now they are being used to oxidize nucleic acids and to induce specific damages to DNA or RNA (*34*). Manganese(III) salen complex was used as catalyst for asymmetric epoxidation of olefins and was the most enantioselective non-enzymatic epoxidation catalyst reported until 1991 (*35*). Chromium(III)-salen complex is an efficient catalyst for the hetero-Diels-Alder reaction and the Nozaki-Hiyama-Kishi reaction with an excellent stereo-selectivity (*36*).



Cobalt Salen



EUK-8, X=H; EUK-134, X=CH_3

Figure 1-5 Structures of common metallo-salen complexes
redrawn from reference (*8*) and (*37*)

Several metallo-salen complexes have been characterized for their biological importance. For example, manganese-salens (EUK-8 and EUK-134, figure 1-5) are known to mimic the metallo-enzymes (superoxide dismutase (SOD) and catalase) that are involved in the removal of reactive oxygen species from cells (*38*)-(*39*). SOD like behavior of Mn-salen allows it to remove superoxide anion by producing oxygen and hydrogen peroxide; and catalase like behavior of Mn-salen allows it to remove hydrogen peroxide by producing water and oxygen (*40*). Studies have also shown that Mn-salen complexes remove reactive nitrogen species (RNS) from the cells indicating the potential role of Mn-salen to reduce damage caused by both oxidative and nitrosative stresses (*37*). Sharpe, et. al. have shown that Mn-salen complexes are oxidized to oxoMn-salen in presence of hydrogen peroxide, peracetate, persulfate, and peroxynitrate (*39*). OxoMn-

salens control the cellular damage due to nitrosative and oxidative stress by rapidly oxidizing NO to NO_2 and nitrite to nitrate (*39*). Non-functional SOD and catalase enzymes are responsible for several diseases that arise due to oxidative stress like Alzheimer's disease, Parkinson's disease, motor neuron disease, and excitotoxic neural injury (*40*). Yang et. al. have shown that Mn-salens reduce the cellular damage caused by Cd exposure by blocking the influx of Cd into cells which subsequently reduces ROS production in cells (*40*).

1.5 Metallo-Salen Complexes as Anticancer Agents

Metallo-salen complexes are a class of chemical nucleases that cleave the DNA and induce apoptosis in cultured human cells (*21*). The flat electronic structure of metallo-salen complexes allows them to interact with DNA, initiate DNA cleavage, damage the DNA, and induce apoptosis in the target cells (*41*). For example, salen complexes of manganese, nickel, cobalt, ruthenium, iron, and copper have been used to induce DNA cleavage in vitro (*42*). Cobalt salen (figure 1-5) can induce apoptosis and inhibit cell proliferation and the activity depends on the type of the bridging substituent (*43*). Salen complexes of different metals have different modes of DNA cleavage. For example, Mn(III)-salen and Ni(II)-salen complexes require terminal oxidants to cleave the DNA whereas Cu(II)-salen and Co(II)-salen complexes require a reducing co-factor to initiate DNA cleavage (*34*). Router et. al. have shown that copper salen cleave the DNA by creating a hydroquinone system that unites with the copper redox system to aid in the spontaneous formation of oxidizing Cu(III) species without the requirement of an activating agent (*34*). Router et. al. have also shown that Cu(II)-salen complex binds to the grooves of DNA double helix and induce single-stranded breaks in the presence of a reducing agent (*42*). Metallo-salen complexes are also known to produce reactive oxygen

species in presence of reducing agents and damage DNA in vitro (*21*). For example, iron and copper salen complexes damage DNA by generating hydroxyl radicals in presence of dithiothreitol; nickel salen complexes require oxidative environment to crosslink the DNA; cobalt salen complexes damage DNA by activating natural oxygen in aqueous solution; manganese salen complexes damage DNA via superoxide dismutase activities; and several metallo-salen complexes are involved in hydrolytic cleavage of DNA and RNA (*21*), (*27*), (*44*). Human cells contain significant levels of the reducing agent glutathione that can be utilized by these compounds to damage DNA (*45*). Ansari et. al. have shown that manganese(III) -salen and -salphen complexes induce apoptosis in MCF7 cells via mitochondrial pathway as indicated by nuclear fragmentation and dislocation of cytochrome c from the mitochondria (*46*). It has also been shown that Fe(III)-salens induce apoptosis in human cancer cells via the mitochondrial pathway (*44*). Ansari et. al., have also shown that salen and salphen complexes of Fe(III) and Mn(III) demonstrate selective cytotoxicity towards cancer cells over normal cells, which is a desirable feature of anticancer drugs (*47*). These findings indicate that these metallo-salen complexes (figure 1-6) show some potential to be used in cancer therapy however; more detailed biochemical and in vivo studies have to be conducted.

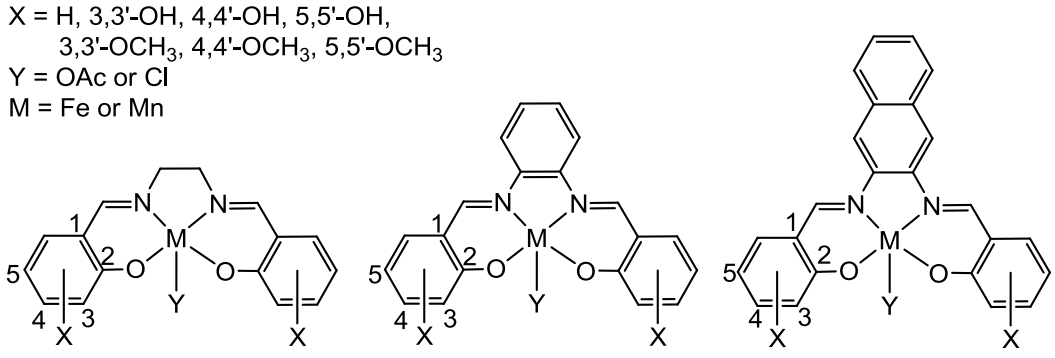


Figure 1-6 Structures of biochemically active metallo-salen derivatives previously synthesized by Mandal lab (*47*) and (*27*)

From the plethora of metal-Schiff base complexes, iron containing Schiff base complexes have been well utilized in biological setting. For example, Ueno et. al. have designed artificial cofactors for myoglobin by replacing Fe(III)(Schiff-base) in the heme site (*48*). Fe(III)-salophene known to exhibit selective cytotoxicity in two different ovarian cancer cell lines that are resistant towards platinum based drugs (*49*). Fe(III)-salphen also exhibit selective cytotoxicity towards tumor cells over normal cells (*27*). Lee et. al. have shown that Fe(III)-salophene can be used to selectively target lymphoma and leukemia cells that are resistant towards other anticancer drugs (*33*).

In an attempt to find a novel anti-tumor drug for the future of cancer therapy six different derivatives of Fe(III)-salphen were synthesized (figure 2-1). Following the synthesis, the cytotoxicity and biological activities of these compounds were evaluated. The results demonstrate that the derivatives of Fe(III)-salphen complexes with 4-CH_3, 4-OCH_3, 4-F, 4-Cl, and 4-Br substituent on the o-phenylenediamine bridge are more toxic to MCF7 and CCL228 cells than the parent salphen (4-H), whereas the derivative with 4-COPh is equally toxic. These metal complexes have their IC_{50} (concentration of a compound at which the cell viability after treatment is 50% as compared to control cells) values in the range of 0.3 to 1.5 μM in two different cell lines which is much lower than that of Cisplatin but the selectivity these compounds is not known. The results also demonstrate that these complexes mediate toxicity via apoptotic pathway as indicated by nuclear fragmentation and caspase-3/7 activation. The toxicity of one of representative compound was inhibited by ascorbic acid indicating that the apoptosis was also induced by oxidative stress. Fe(III)-salphen derivatives with very low IC_{50} values establish some potential as a chemotherapeutic drug.

Chapter 2

Synthesis of Novel Metallo-Salphen Derivatives and Evaluation of their Anti-Tumor Activities

2.1 Introduction

There is an emerging need for the synthesis of novel anticancer drugs that can overcome Cisplatin resistance, target diverse range of tumors, have minimal side effects, and that can be easily transported to the tumor site while having high specificity towards cancer cells. Small molecule drugs are preferred over macro molecules because their small size that allows them to easily reach the target cells without the need of an artificial transport system. Metallo-salen complexes are a class of small molecule based drugs that have been studied for their anticancer properties. Due to the ease of manipulation and synthesis, several different metal coordinated complexes of salen have been synthesized and tested for their cytotoxicity. Most of the metallo-salens can generate free radicals in the body that damage the DNA and kill the cells by mediating oxidative stress. However, more work needs to be done in this field to isolate some drugs with fewer side effects and known mode of action.

In this context, a set of six different derivatives of Fe(III)-salphen (compound 1, H) were synthesized by varying the group-X at position-4 on the o-phenylenediamine bridge (figure 2-1). These compounds were then characterized by Infrared (IR) spectroscopy and elemental analysis before investigating their biological properties. Cytotoxicity assay of these compounds was performed along with the parent salphen (known to mediate toxicity via apoptosis in cancer cells) and the results showed that the

IC_{50} values of these compounds are much lower than Cisplatin and also lower than the parent compound in most cases (table 2-2).

2.2 Materials and Methods

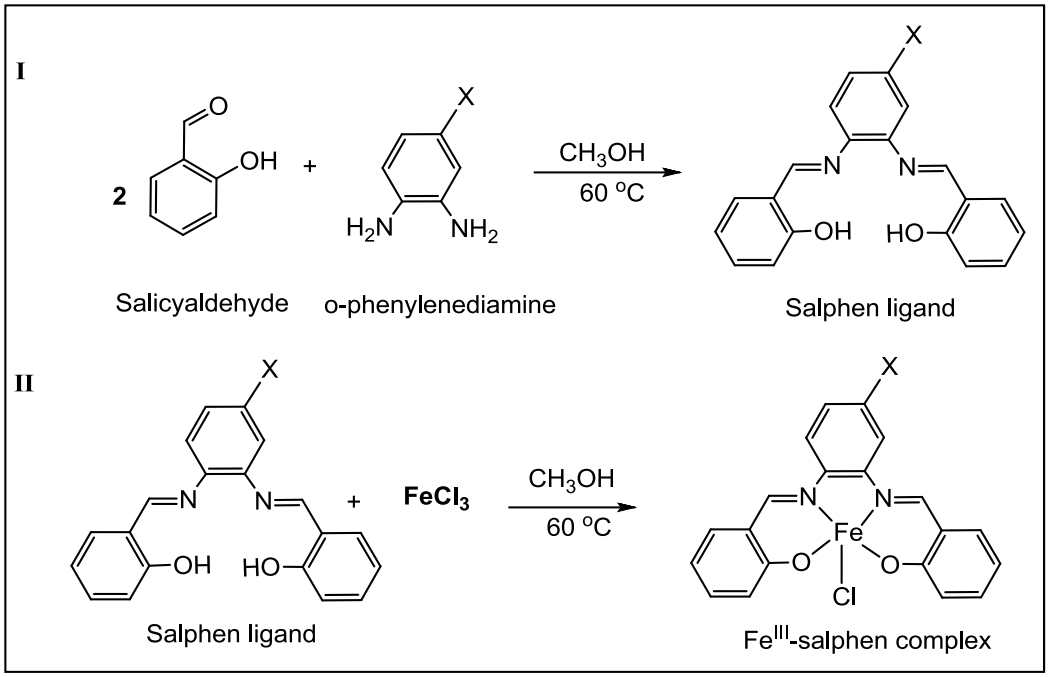
2.2.1 Reagents

All reagents and buffers were purchased from Sigma-Aldrich unless specified. Tissue culture medium DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum), streptomycin, and penicillin were purchased from Sigma-Aldrich. Anhydrous ferric chloride was purchased from Spectrum Chemical Manufacturing Corporation, DAPI (4′, 6-diamidino-2-phenylindole) was purchased from Invitrogen, and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was purchased from Tokyo Chemical Industry Co. CCL228 and MCF7 cell lines were purchased from ATCC (American Type Culture Collection). Perkin Elmer Model 2400 CHN analyzer was used for elemental analyses and Bruker Vector 22 infrared spectrometer with KBr cell was used to obtain the FT-IR spectra. Fluostar-Omega, BMG, Labtech micro-plate reader was used to record the absorbance of 96 well titer plates following MTT assay.

2.2.2 Synthesis of Fe(III)-salphen and their derivatives (compounds 1-7)

Fe(III)-salphen derivatives were synthesized and characterized following a common procedure that was previously described by Mandal lab (*50*). As shown in scheme 2-1, ligands were synthesized by reacting salicylaldehyde with the corresponding o-phenylenediamine derivative in warm methanol (step 1). The ligands were reacted with one equivalent of anhydrous ferric chloride in methanol by stirring it over 60 °C water bath for 15 min and cooled down to room temperature overnight to precipitate out metal-complexes from the reaction mixture (step 2). The ligands precipitated out as colorful crystals (orange, yellow, light brown) and the metal complexes precipitated out as dark

brown to black crystals that were slightly soluble in methanol. They were recrystallized from methanol and the yields of these reactions were in the range of 50% to 60%. Following the synthesis, they were characterized by elemental analysis and Infrared (IR) spectroscopy. The observed values of C, H, and N composition of all the compounds were comparable to the calculated values. Results of CHN analysis showed the presence of water in most of these compounds.



Scheme 2-1 Synthesis of Fe(III)-salphen derivatives
X = H, CH_3, OCH_3, F, Cl, Br, or COPh

Synthesis of Fe [N,N'-bis(salicylidene)-o-phenylenediamine]•Cl (compound 1, H)

The synthesis of this compound was reported previously by Mandal lab (*27*). In brief, two equivalent of salicylaldehyde (1.23 g, 10 mmol) was mixed with one equivalent of o-phenylenediamine (541 mg, 5 mmol) in warm methanol to obtain a precipitate of salphen ligand. 475 mg (1.5 mmol) of this ligand was complexed with 246 mg (1.5 mmol) of ferric chloride in warm methanol to obtain 1 (compound 1, H).

Synthesis of Fe [N,N'-bis(salicylidene)-4-Methyl-o-phenylenediamine]•Cl (compound 2, CH_3)

Two equivalent of salicylaldehyde (1.22 g, 10 mmol) was mixed with one equivalent of 4-Methyl-o-phenylenediamine (611 mg, 5 mmol) in warm methanol to obtain a precipitate of 4-Methyl salphen ligand. 496 mg (1.5 mmol) of this ligand was complexed with 244 mg (1.5 mmol) of ferric chloride in warm methanol to obtain 2 (compound 2, CH_3)

Synthesis of Fe [N,N'-bis(salicylidene)-4-Methoxy-o-phenylenediamine]•Cl (compound 3, OCH_3)

Two equivalent of salicylaldehyde (612 mg, 5 mmol) was mixed with one equivalent of 4-Methoxy-o-phenylenediamine (346 mg, 2.5 mmol) in warm methanol. Crystals of 4-Methoxy salphen ligand could not be isolated as it was highly soluble in methanol. 487 mg (3 mmol) of ferric chloride was added to the reaction mixture to obtain 3 (compound 3, OCH_3).

Synthesis of Fe [*N*,*N*'-bis(salicylidene)-4-Fluoro-o-phenylenediamine]•Cl (compound 4, F)

Two equivalent of salicylaldehyde (1.22 g, 10 mmol) was mixed with one equivalent of 4-Fluoro-o-phenylenediamine (630 mg, 5 mmol) in warm methanol to obtain a precipitate of 4-Fluoro salphen ligand. 502 mg (1.5mmol) of the ligand was complexed with 245 mg (1.5 mmol) ferric chloride in warm methanol to obtain 4 (compound 4, F).

Synthesis of Fe [*N*,*N*'-bis(salicylidene)-4-Chloro-o-phenylenediamine]•Cl (compound 5, Cl)

Two equivalent of salicylaldehyde (1.23 g, 10 mmol) was mixed with one equivalent of 4-Chloro-o-phenylenediamine (712 mg, 5 mmol) in warm methanol to obtain

a precipitate of 4-Chloro salphen ligand. 526 mg (1.5 mmol) of the ligand was complexed with 244 mg (1.5 mmol) Fe(III) chloride in warm methanol to obtain 5 (compound 5, Cl).

Synthesis of Fe [*N*,*N*'-bis(salicylidene)-4-Bromo-o-phenylenediamine]•Cl (compound 6, Br)

Two equivalent of salicylaldehyde (733 mg, 6 mmol) was mixed with one equivalent of 4-Bromo-o-phenylenediamine (561 mg, 3 mmol) in methanol to obtain a precipitate of 4-Bromo salphen ligand. 593 mg (1.5 mmol) of the ligand was complexed with 244 mg (1.5 mmol) of Fe(III) chloride in warm methanol to obtain 6 (compound 6, Br).

Synthesis of Fe [*N*,*N*'-bis(salicylidene)-3,4-diaminobenzophenone]•Cl (compound 7, COPh)

Two equivalent of salicylaldehyde (1.23 g, 10 mmol) was mixed with one equivalent of 3,4-Diaminobenzophenone (1.07 g, 5 mmol) in methanol to obtain a precipitate of 4-Benzophenone salphen ligand. 631 mg (1.5 mmol) of the ligand was complexed with 244 mg (1.5 mmol) Fe(III) chloride in methanol to obtain 7(compound 7, COPh).

2.2.3 MTT Assay and IC_{50} Determination

Human colon cancer (CCL228) and breast cancer (MCF7) cells were grown and maintained in cell culture medium prepared from Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat inactivated fetal bovine serum (FBS, 10%), L-glutamine (1%), and penicillin/streptomycin (0.1%). Cells were grown in 96 well micro-titer plates and were incubated in humidified incubator with 5% CO_2 at 37°C.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of Fe(III)-salphen and its derivatives. MTT assay was

performed using the procedure that was described previously by Mandal lab (*47*). CCL228 and MCF7 cells seeded into 96 well titer plates (180 µL DMEM) were grown to 60-70% confluency and were treated with 20 µL of compounds 1-7 dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0-2 µM for 96 hours. Control cells were treated with the same amount of DMSO and 180 µL DMEM mixed with 20 µL DMSO was used as a blank. Treatments were done at six different concentrations with eight replicates for each of the concentrations. MTT assay was performed after 96 hours of treatment. For the MTT assay, 20 µL MTT (stock 5 mg/mL in PBS) was added into each well of the titer plate and incubated for 2 hours under normal growth conditions to allow the viable cells to convert MTT (yellow) to formazan (purple). After 4 hours, the media was discarded and formazan (insoluble in media) was dissolved in 100 µL of DMSO and incubated for 2 hours on top of a shaker. Absorbance readings of the 96 well titer plate were then recorded at 560 nm using a titer plate reader. The percentage of viable cells relative to the control was calculated based on the absorbance reading (higher absorbance signifies more formazan production by higher number of viable cells) and the viability curve was obtained by plotting absorbance against the concentration. IC_{50} was determined from the viability curve as concentration of the compound at which the amount of MTT to formazan conversion by viable cells was 50% compared to control cells. Each treatment was done with 8 replicates and the entire experiment was repeated twice to reconfirm the result.

2.3 Results and Discussion

2.3.1 Synthesis and characterization of Fe(III)-salphen derivatives

Six different derivatives of the parent Fe(III)-salphen chloride (compound 1, H) were synthesized by altering the group-X at position-4 on the o-phenylenediamine bridge

(figure 2-1) to evaluate their cytotoxicity and biological significance. Salphen ligands were synthesized by reacting two equivalents of salicylaldehyde with the corresponding o-phenylenediamine derivative. The ligands were then reacted with one equivalent of anhydrous ferric chloride to yield Fe(III)-salphen derivatives (compounds 2-7, figure 2-1) as described in chapter 2.2.2 (scheme 2-1). The compounds were then characterized by elemental analysis and Infrared (IR) spectroscopy and the results are shown in table 2-1. The observed values of C, H, and N composition of all the compounds were comparable to the calculated values. Results of CHN analysis showed the presence of water in most of these compounds.

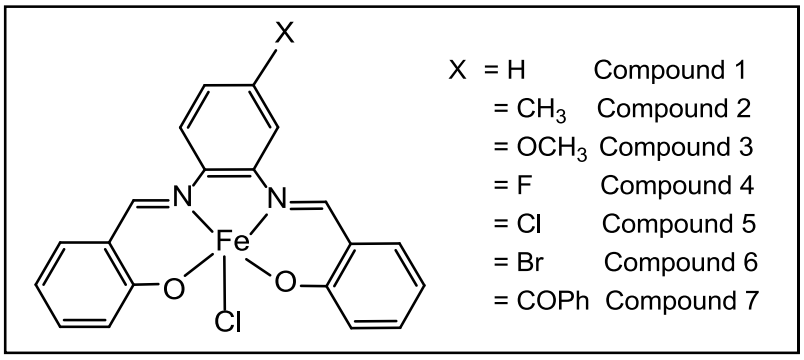
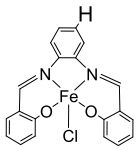
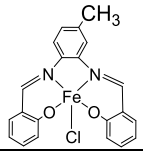
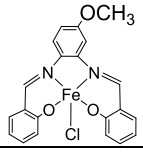
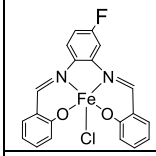
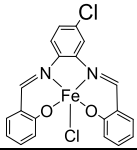
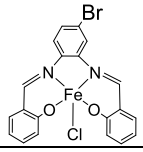
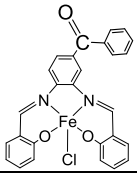


Figure 2-1 General structure of Fe(III)-salphen derivatives (compounds 1-7)

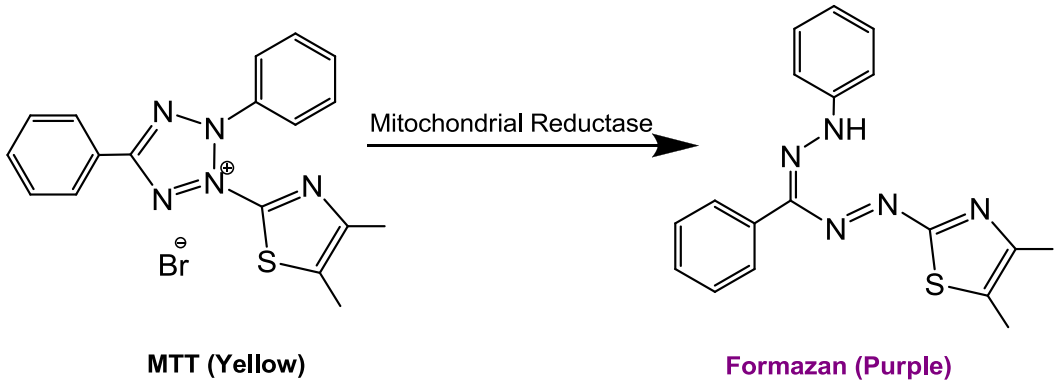
Table 2-1 Characterization of Fe(III)-salphen derivatives

Compound	Formula	CHN (%)	FT-IR (KBr, cm^{-1})
1* 	$C_{20}H_{14}N_2O_2FeCl$ 2.5 H_2O	Calculated - C:53.37%; H:4.26%; N:6.22% Observed – C:53.32%; H:3.88%; N:6.00%	1600, 1590, 1530, 1450, 1430, 1380, 1320, 1200, 1140, 1110, 910, 880, 850, 800, 730, 600, 530
2 	$C_{21}H_{16}N_2O_2FeCl$ 0.2 H_2O	Calculated: C:59.58%; H:3.91%; N:6.62% Observed - C:59.48%; H:3.89%; N:6.58%	1607, 1579, 1535, 1462, 1440, 1373, 1316, 1196, 1144, 914, 818, 761, 610, 552, 504
3 	$C_{21}H_{16}N_2O_3FeCl$ 1 H_2O	Calculated - C:55.60%; H:4.00%; N:6.17 % Observed – C:55.54%; H:5.60%; N:5.97%	2940, 2605, 2497, 1607, 1535, 1378, 1313, 1262, 1189, 1150, 1034, 821, 758, 606, 531
4 	$C_{20}H_{13}N_2O_2FFeCl$ 0.5 H_2O	Calculated - C:55.52%; H:3.27%; N:6.48% Observed - C:55.27%; H:3.36%; N:6.60%	1609, 1578, 1558, 1531, 1497, 1377, 1261, 1144, 982, 917, 823, 761,610, 550, 504
5 	$C_{20}H_{13}N_2O_2ClFeCl$ 1.5 H_2O	Calculated - C:51.42%; H:3.45%; N:6.00% Observed – C:51.15%; H:3.07%; N:6.24%	1602, 1575, 1532, 1461, 1374, 1320, 1195, 1145, 935, 904, 816, 761, 604, 548
6 	$C_{20}H_{13}N_2O_2BrFeCl$ 1.9 H_2O	Calculated - C:46.30%; H:3.27%; N:5.40% Observed - 46.58%C, 3.57%H, 5.26%N	1602, 1572, 1532, 1461, 1373, 1313, 1194, 1149, 928, 894, 814, 760, 602, 547
7 	$C_{27}H_{18}N_2O_3FeCl$ 1.3 H_2O	Calculated - C:60.70%; H:3.89%; N:5.24% Observed - C: 60.58%; H: 4.12%; N: 5.43%	3059, 1658, 1604, 577, 532, 1441, 1374, 1315, 1272, 1196, 1129, 814, 712, 662, 550.

1* previously synthesized by Mandal lab (*50*)

2.3.2 Fe(III)-salphen derivatives are toxic to human cancer cells

MTT assay was used to evaluate the cytotoxicity of Fe(III)-salphen derivatives in CCL228 and MCF7 cell lines. MTT assay is a colorimetric assay that evaluates the mitochondrial activity of cells based on the capability of live cells to convert yellow colored dye MTT to purple colored formazan as shown in scheme 2-2 (*51*).



Scheme 2-2 Conversion of MTT to formazan in the mitochondria of live cells

Cells were incubated with different concentrations (0-2 μM) of compounds 1-7 for 96 hours in a 96 well titer plate after which MTT assay was used to access percent cell viability relative to the control cells as explained in chapter 2.2.3. The viability curve that was used to determine the IC_{50} values for compounds 1-7 (CCl228 cells) is shown in figure 2-2 and the IC_{50} values with standard error of mean (SEM) for Cisplatin and compounds 1-7 are summarized in table 2-2.

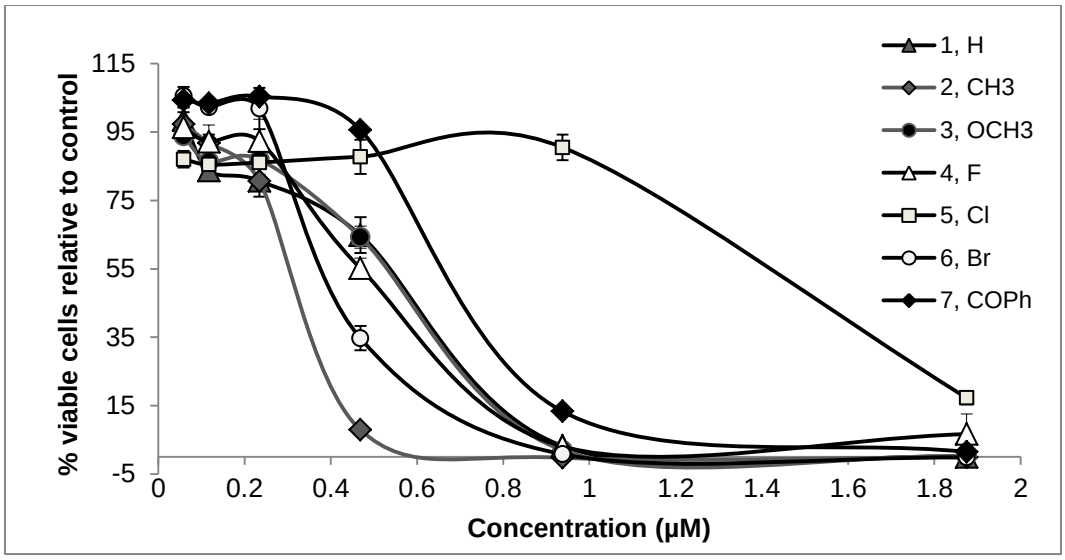


Figure 2-2 Fe(III)-salphen derivatives are toxic to human colon cancer cells.

A single layer of CCL228 cells grown up to 60-70% confluent in 96 well micro titer plate were treated with different concentrations (0 to 2 μM) of Fe(III)-salphen derivatives for 96 h. Cell viability was measured by MTT assay and the percentage of viable cells relative to DMSO treated control was plotted as a function of concentration. Each treatment was done with 8 replicates and was repeated twice. The lines represent the viability curves and the error bars represent standard error of the mean.

Table 2-2 IC_{50} values of Fe(III)-salphen derivatives towards cancer cell lines.

Compounds	**IC_{50} (μM)**	
	CCL228	**MCF7**
Cisplatin*	29 (±3.5)*	18 (±2.3)*
1, H	0.56 (±0.02)	1.30 (±0.10)
2, CH_3	0.32 (±0.02)	0.31 (±0.01)
3, OCH_3	0.56 (±0.01)	0.67 (±0.01)
4, F	0.50 (±0.02)	0.46 (±0.01)
5, Cl	1.48 (±0.03)	0.32 (±0.16)
6, Br	0.40 (±0.01)	0.46 (±0.02)
7, COPh	0.68 (±0.10)	1.34 (±0.14)

The number in parenthesis indicates the standards error of the mean (SEM)
*The IC_{50} values of Cisplatin were obtained from reference (*47*).

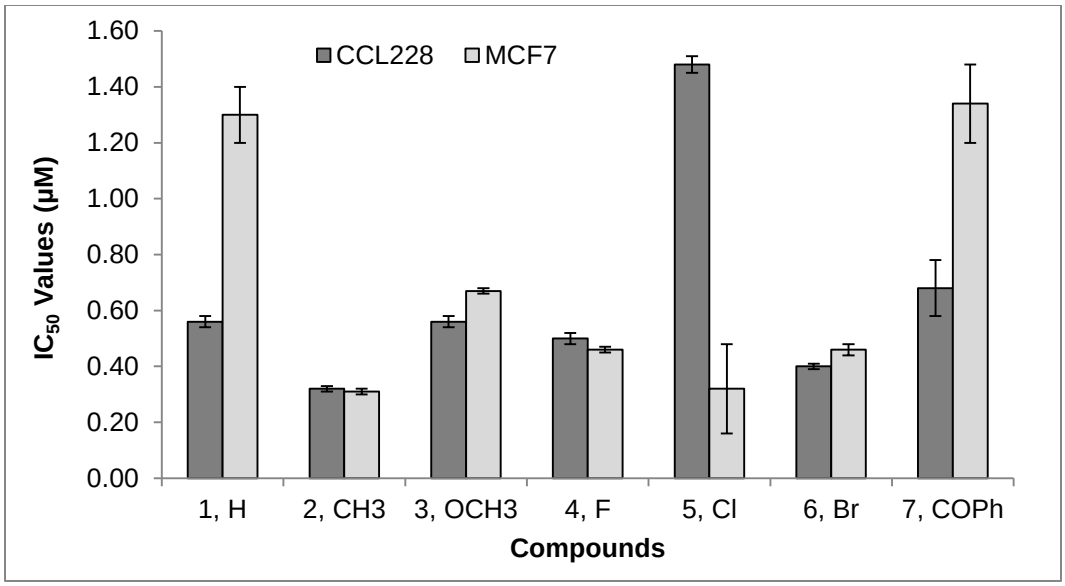


Figure 2-3 IC_{50} values of Fe(III)-salphen derivatives towards cancer cell lines

The IC_{50} values of Fe(III)-salphen derivatives (table 2-2) are represented as a bar graph. The error bars represent the standard error of the mean (SEM).

The result showed that Fe(III)-salphen derivative are toxic to both MCF7 and CCL228 cells with very low IC_{50} values (table 2-2). IC_{50} values of compounds 1-6 towards MCF7 cells are lower than 0.7 µM and that for compound 7 was 1.34 µM. In CCL228 cells, IC_{50} values of compounds 1-4 and 6-7 are below 0.7 µM and that of 5, Cl was 1.48 µM (table 2-2). Compound 2 (2, CH_3) was most toxic to both MCF7 and CCL228 cells with IC_{50} values of 0.31 µM and 0.32 µM respectively (Cisplatin 18 µM and 29 µM)

2.4 Summary and Conclusion

The results suggest that the Fe(III)-salphen derivatives are potential inhibitors of cell growth and proliferation. The nature of the substituent on the o-phenylenediamine bridge slightly altered the toxicity levels which could be due to differences in cellular uptake. Among the six different derivatives of Fe(III)-salphen (compound 2, CH_3) was the

most toxic to both the cell lines with IC_{50} values of 0.31 µM in MCF7 cells and 0.32 µM in CCL228 cells. Changing the substituents at position-4 on the o-phenylenediamine bridge did not show any significant decrease in the IC_{50} values of compounds 1-7 towards CCL228 cells but up to 4 fold decrease in the IC_{50} values was observed in MCF7 cells (figure 2-3). Although compound 7 (7, COPh) has increased aromaticity it did not show higher toxicity towards MCF7 and CCL228 cells as reported previously (*21*) which could be due to decreased cellular uptake. Low IC_{50} value is one among the long list of desirable features for a compound to be used as a drug. This suggests that these compounds show some potential as anticancer drugs but their biochemical and in vivo characterization is required.

Chapter 3

Biochemical Mechanism of Metallo-Salphen Mediated Toxicity

3.1 Introduction

Metallo-salphens are a class of chemical nucleases that can modify the genetic material because of their oxidative nature. Metallo-salphens are considered to be potential anti-tumor drugs because they are highly toxic to cancer cells with low IC_{50} values. Some metallo-salphen complexes are even known to exhibit tumor selective apoptosis. For example, Mn(III)-salphen derivatives have shown 3-5 fold selective toxicity towards cancer cells over normal cells (*47*). Metallo-salen complexes are also known to produce reactive oxygen species (ROS) and damage DNA in vitro *(12)* which in turn can lead to apoptotic cell death. Almost all anticancer drugs work by inducing apoptosis in the target cells but very few are able to selectively target tumor cells. Several side effects associated with chemotherapy arise due to non-specificity of anticancer drugs. Cisplatin and many other drugs that are widely used for chemotherapy have severe side effects and suffer from acquired resistance. Scientists are continuously synthesizing new compounds and screening them for their cytotoxicity with in an attempt to discover a novel drug that can overcome the drawbacks of existing anticancer drugs. However, only few of them get characterized for their biochemical activities, fewer are studied in vivo, and only handful of them enter clinical trials.

Several metallo-salphen derivatives have been synthesized and screened for their toxicity in human cells but only few have been explored in detail to elucidate their biochemical mechanisms. Detailed biochemical analysis and in vivo studies are essential before considering any compound as a potential drug. Mandal lab have previously shown

that Fe(III)-salen and -salphen induce apoptosis via mitochondrial pathway (*27*) and here the study is expanded to six different derivatives of Fe(III)-salphen in an attempt to find a novel metallo-salphen derivative with favorable anticancer properties. Some possible mechanisms that might be involved in the cytotoxicity were investigated. The results show that Fe(III)-salphen derivatives induce apoptosis as characterized by condensed and fragmented nucleus observed after DAPI staining. Activation of the executioner caspases-3/7 is another confirmation to prove these compounds induce apoptosis which is most likely due to mitochondrial pathway as shown by Ansari et. al. in case of compound 1(1, H) (*27*). The apoptosis induced by these compounds was inhibited by ascorbic acid, an antioxidant, suggesting that apoptosis is due to oxidative stress via generation of reactive oxygen species and free radicals.

3.2 Materials and Methods

3.2.1 DAPI staining of the nucleus

DAPI staining was performed as described previously by Mandal laboratory (*47*). CCL228 cells were grown on cover slips in a 5mL plate for up to 60% confluency and treated with 5 µM of Fe(III)-salphen derivatives (compounds 1-7) dissolved in DMSO for 16 hours. Control cells were treated with an equal amount of DMSO and Cisplatin was used as a positive control. Cells were fixed with 4% formaldehyde in phosphate buffer saline (PBS) for 15 min at room temperature and washed with PBS to remove excess formaldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and washed three times with cold PBS. Cells were briefly incubated with 5 mg DAPI at room temperature and washed three times with cold PBS to remove excess staining. DAPI stained cells were mounted on a microscope slide using mounting media (Vectashield H-1000, Vector lab) and were visualized under blue filter of a fluorescence microscope

(figure 3-2). DAPI staining was also performed on CCL228 cells treated with 1 µM of compound 2 (2, CH_3) and on control cells treated with DMSO (figure 3-3).

3.2.2 Caspase-3/7 activity assay

Caspase-3/7 assay was performed as described previously by Mandal lab (*47*). CCL228 cells were treated with 5 µM Fe(III)-salphen (1, H) and one of its derivatives (compound 2, CH_3) for 16 hours. Compound 1 (1, H) was used as a positive control as it was previously shown to activate caspase-3/7 (*50*). Cells were harvested, lysed, and centrifuged at 2500 g for 10 min at 4°C to isolate whole cell extract. 150 µL of 1 µg/µL of the whole cell extract was mixed with 50 µL of buffer containing caspase-3/7 substrate and was incubated for 15 min at 37°C as described in the protocol for SensoLyte TM Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec Inc.CA) (*47*). The fluorescence intensity was measured at excitation wavelength of 354 nm and emission wavelength of 442 nm every 10 min for 2 hours. The concentration of activated caspase-3/7 was determined by using the reference standard curve and was represented as a bar graph (figure 3-4). Each treatment was done with 4 replicates.

3.2.3 Ascorbic acid assay

CCL228 cells were grown to 60-70% confluency in a 96 well titer plate were treated with 1 µM of compounds 1 and 2 (1, H and 2, CH_3) in presence of increasing concentration (0-200 mM) of ascorbic acid. The control cells were treated with 1 µM of compounds 1 and 2. The cell viability was measured after 96 hours using MTT assay as described in section 2.2.3. The percent viable cells relative to the control were plotted against the concentration of ascorbic acid to obtain the plot in figure 3-5. Each treatment was done with 4 replicates.

3.2.4 RNA extraction, cDNA synthesis, and PCR

Cells were grown in 5 ml of DMEM media in 60 mm plates until they were 60% confluent. The cells were treated with 1 µM compound 2 (2, CH_3) or with 15 µM Cisplatin. In both cases, the control cells were treated with equal amount of DMSO. Following the treatment, the cells were incubated for 2, 4, 8, and 16 hours after which they were harvested and subjected to RNA extraction, cDNA (complimentary DNA) synthesis, and polymerase chain reaction (PCR) as described previously by Mandal lab (*52*). The cells were centrifuged at 500g for 5 min at 4 °C, the supernatant was discarded, the cell pellet was re-suspended in DEPC (diethyl- pyrocarbonate) treated buffer A (20 mM Tris–HCl, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF (phenylmethylsulfonyl fluoride)), and incubated for 10 min on ice. It was then centrifuged at 3500 g for 5 min at 4 °C and the supernatant was subjected to phenol-chloroform extraction. The aqueous layer was then used for ethanol precipitation for 3 hours in -80 °C after which it was centrifuged at 13000 g for 15 min and the ethanol was discarded to leave behind RNA pellet. The RNA pellets were air dried, dissolved in DEPC treated water containing 1 mM ethylenediaminetetraacetic acid (EDTA), and quantified using nanodrop spectrophotometer. In the phenol-chloroform extraction, proteins remain in the organic phase while nucleic acids remain in the aqueous phase. RNA can be separated out in the aqueous phase by adjusting the pH of the aqueous phase.

For cDNA synthesis a 25 µL mixture of the following reagents were prepared: 2.4 µM oligo dT (Promega), 100 units of MMLV reverse transcriptase (Promega), 1 × MMLV RT buffer (Promega), 100 µM of dNTPs (dATP, dGTP, dCTP, and dTTP, Promega), 1 mM DTT, 20 units of RNaseOut (Invitrogen), and 500 ng of the RNA extract. This reaction mixture was then used to perform reverse transcription reaction. The product of cDNA synthesis was diluted to 100 µL by adding ultrapure water and was

stored in 4 °C. 5 µL of the diluted cDNA was used for PCR reaction (30 cycles) using product specific primer pairs listed in table 3-1. The PCR product was loaded onto 1.5% agarose gel and was subject to gel electrophoresis (140 V). The gel images were taken using Alpha Imager instrument. Each experiment was repeated three times.

Table 3-1 Nucleotide sequence of primers used in PCR analysis

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
Cyclin A	AAGAAGCAGCCAGACATCACGGAA	AGCTGCAGTTTCCCTCTCAGAACA
Cyclin B	TTGATACTGCCTCTCCAAGCCCAA	TTGGTCTGACTGCTTGCTCTTCCT
Cyclin D	AGAAGCTGTGCATCTACACCGACA	TGATCTGTTTGTTCTCCTCCGCCT
Cyclin E	TTTCAGGGTATCAGTGGTGCGACA	ACAACATGGCTTTCTTTGCTCGGG

3.3 Results and Discussion

3.3.1 Fe(III)-salphen derivatives are toxic to CCL228 cells

Cells in culture are said to be healthy if they are growing in monolayer and have a distinct smooth morphology. When treated with toxic compounds cells become sick and undergo apoptosis as characterized by shrinkage, aggregation, and formation of cell debris. To examine the toxic effect of Fe(III)-salphen derivatives on the morphology, CCL228 cells were treated with 5 µM of compounds 1-7 for 24 h after which the cells were observed under differential interference contrast (DIC) settings of fluorescence microscope. It can be seen that compounds 1-7 with very low IC_{50} (nM to µM range) induced shrinkage, clumping, rounding up, and degradation of the treated cells (figure 3-1). Cisplatin was also efficient to induce distinct morphological changes in the treated cells as compared to untreated healthy cells in control panel (figure 3-1). These results

further support that the Fe(III)-salphen derivatives are toxic to cancer cells and induce cell death.

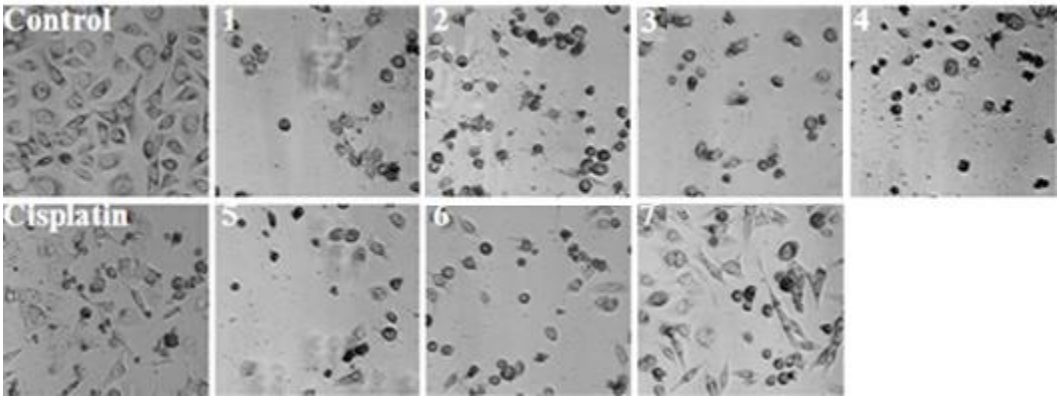


Figure 3-1 Effect of Fe(III)-salphen derivatives on cell growth and morphology.

CCL228 cells were treated with DMSO in control panel and 5 μM of Fe(III)-salphen derivatives in panels 1-7 for 24 h. Cell morphology was analyzed under differential interference contrast (DIC) settings of fluorescence microscope. Cisplatin was used as a positive control. Numbers on each panel represent the corresponding Fe(III)-salphen derivatives (1-H, 2-CH_3, 3-OCH_3, 4-F, 5-Cl, 6-Br, and 7-COPh).

3.3.2 Fe(III)-salphen derivatives induce apoptosis

Apoptosis (programmed cell death) is essential to maintain the balance between cell proliferation and cell death. Apoptosis removes the sick and unwanted cells to maintain the normal physiology of the body. Several internal and external stimuli that are harmful to the cells can initiate apoptosis. Almost all anticancer drugs induce apoptosis in cancer cells to control unwanted proliferation (*22*). Biochemical modifications like protein cleavage, protein-crosslinking, DNA fragmentation, and nuclear condensation are known to be exhibited by apoptotic cells (*28*).

In order to investigate if apoptosis is involved in Fe(III)-salphen derivatives mediated toxicity, the effect of Fe(III)-salphen derivatives on nuclear integrity was analyzed by staining the treated cells with DAPI (4',6-diamidino-2-phenylindole). Nucleus of live cells or that of fixed cells can be observed under a fluorescence microscope by

staining it with DAPI. DAPI is a dye that strongly binds to the A-T rich regions of the DNA and can cross the membrane of both live and dead cells. Thus, DAPI can be used to stain the nucleus of both live and dead cells. DAPI staining was performed on CCL228 cells treated with 5 μM of Fe(III)-salphen derivatives (compounds 1-7), control cells treated with an equal amount of DMSO, and on positive control cells treated with Cisplatin after fixing them with formaldehyde. DAPI stained cells were excited with ultraviolet radiation (360 nm) and its emission maximum (460 nm) was detected through a blue filter. The results showed that the control cells are evenly stained with DAPI and have an intact nucleus whereas the treated cells stained unevenly. More intense DAPI staining corresponds to the condensed region of the nucleus and the unstained region corresponds to the space between the disintegrating nucleus. Compounds 2 and 5 (2,CH_3 and 5, Cl) had the most detrimental effect on the nuclear morphology and resulted in more severe condensation and fragmentation of the nucleus (figure 3-2). Likewise, compounds 1, 3, 4, 6, 7, and Cisplatin also had some degree of nuclear condensation and fragmentation. The condensation and fragmentation of nucleus along with the formation of ring like structure in the nucleus (almost all treated cells) indicate that the Fe(III)-salphen derivatives and Cisplatin all induce apoptotic cell death.

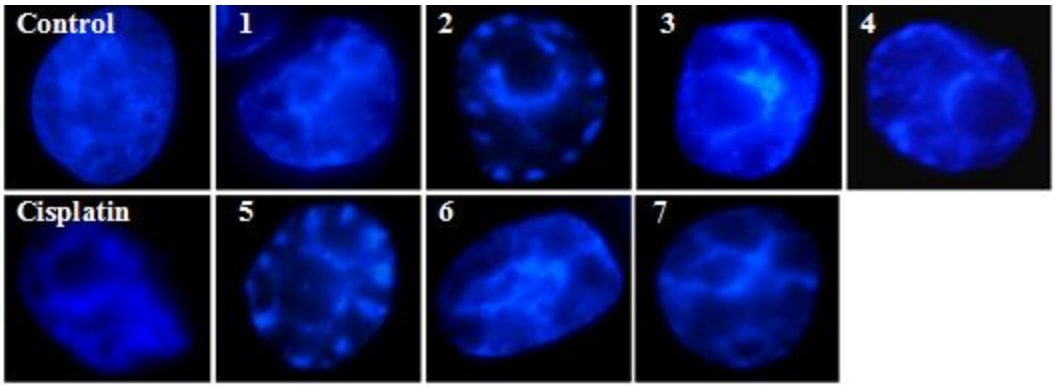


Figure 3-2 Effect of Fe(III)-salphen derivatives on nuclear integrity of CCL228 cells.

CCL228 cells were grown up to 60% confluency and treated with 5 μM of Fe(III)-salphen derivatives for 16 h. Cells were fixed with formaldehyde and stained with DAPI after which they were observed under a fluorescence microscope. Control cells were treated with equal amount of DMSO and Cisplatin was used as a positive control. Numbers on each panel represent compounds 1-7 (1-H, 2-CH_3, 3-OCH_3, 4-F, 5-Cl, 6-Br, and 7-COPh) respectively. Dr. Sahba Kasiri helped me to obtain these pictures by fluorescence microscopy.

Compound 2 (4-CH_3 Fe(III)-salphen) was the most toxic with IC_{50} value of 0.32 (±0.02) μM and showed significant amount of nuclear disintegration so it was further used to study the biochemical properties. In order to investigate the time point dependence on the nuclear morphology, DAPI staining was performed on CCL228 cells treated with 1 μM of compound 2 (2, CH_3) and control cells treated with equal amount of DMSO for 24 h, 48 h, and 72 h. When compared to the control cells, all treated cells showed condensed and disintegrated nucleus as shown by uneven dark and intense blue regions in the bottom three panels (figure 3-3). The intense blue region in the control 24 h panel is not due to apoptosis, it represents a dividing cell with condensed nucleus during telophase. In the treated cells, nuclear condensation and fragmentation increased over time and by 96 h the nucleus was completely disintegrated and no DAPI stain could be detected (result not shown). Figure 3-2 (panel 2) and figure 3-3 together show that compound 2 (2, CH_3)

effectively induces nuclear fragmentation and apoptosis and completely kills the cells by day 4 due to the high levels of toxicity.

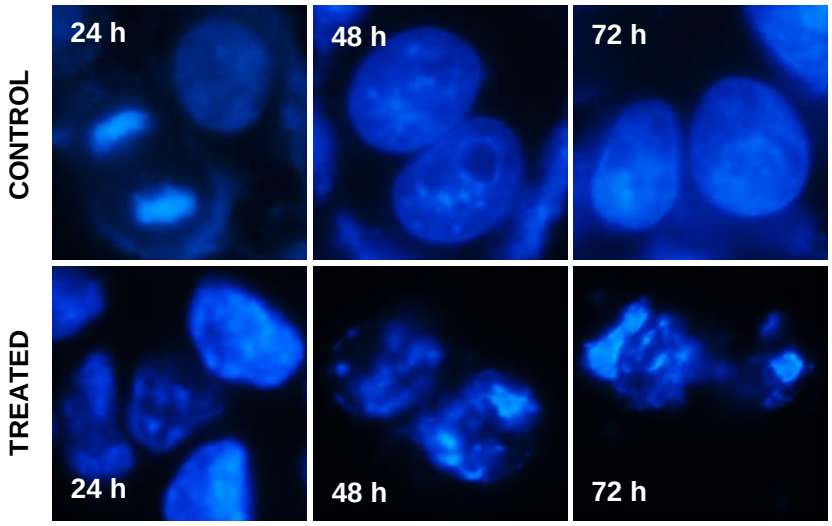


Figure 3-3 Time dependent effect of 4-CH_3 Fe(III)-salphen on nuclear integrity.

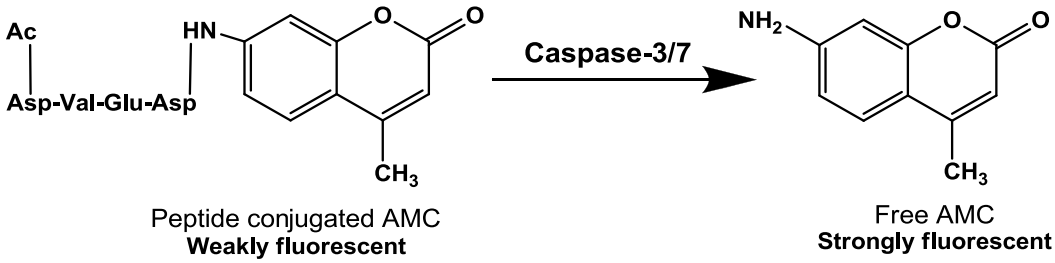
CCL228 cells were grown up to 60% confluency and treated with 1 μM of compound 2 (2, CH_3) for 24 h, 48 h, and 72 h. Cells were fixed with formaldehyde and stained with DAPI after which they were observed under a fluorescence microscope. Control cells were treated with equal amount of DMSO.

3.3.3 Fe(III)-salphen derivatives activate caspases-3/7

Both extrinsic and intrinsic pathways of apoptosis end at an execution phase in which the executioner caspases-3, -6, and -7 are activated (figure 1-4) (*28*). Caspase-3 can be activated by caspase-8, caspase-9, or caspase-10, so it is an important executioner caspase involved in both the pathways of apoptosis (*28*). In the mitochondrial pathway, caspase-9 cleaves and activates the executioner caspases which in turn activates the endonucleases and proteases that initiate apoptosis (*28*).

The results of DAPI staining suggested that Fe(III)-salphen derivatives induce apoptotic cell death in CCL228 cells. An increase in level of caspace-3 after the treatment should observed if apoptosis is involved in Fe(III)-salphen derivative mediated toxicity. To confirm that the cell death was due to apoptosis, caspase-3/7 assay was performed on

control CCL228 cells and CCL228 cells treated with 5 µM compounds 1 and 2 for 16 hours. If active caspase-3/7 is present in the whole cell extract being analyzed then the peptide is cleaved off from the peptide-coumarin conjugate Ac-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin) to generate strongly fluorescent free AMC (scheme 3-1) that can be detected at excitation wavelength of 354 nm and emission wavelength of 442 nm (*53*). The concentration of activated caspase-3/7 was determined by using the reference standard curve and was presented as a bar graph (figure 3-4).



Scheme 3-1 Protelytic cleavage of peptide-coumarin conjugate by caspase-3/7

As expected, threefold increase in the level of caspase-3/7 activity was observed after the cells were treated with 5 µM compounds 1 and 2 as compared to the untreated control (figure 3-4). Compound 1 (1, H) (at 5 µM) showed threefold increase in the level of caspase-3/7 activity (figure 3-4) which is less than sevenfold increase (at 100 µM) reported before (*27*). This difference observed in the caspase-3/7 levels for compound 1 (1, H) could be due to the concentration dependence of apoptosis. Although compounds 3-7 were not examined for caspase-3/7 assay, they most likely exhibit similar results with slight variances due to the structural similarities. These results further support the involvement of apoptotic pathway in the Fe(III)-salphen (and its derivatives) mediated toxicity in CCL228 cells.

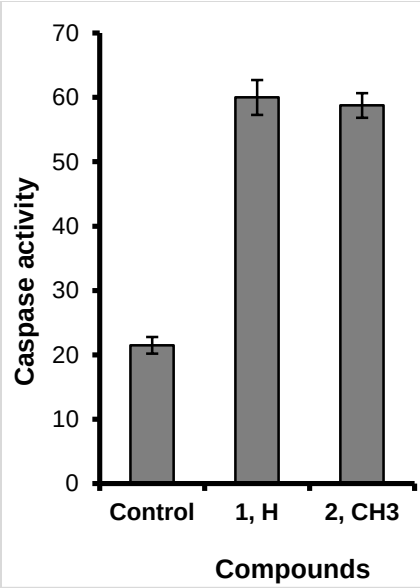


Figure 3-4 Effect of Fe(III)-salphen derivatives on caspase 3/7 activation.

CCL228 cells were treated with 5 µM of compounds 1 and 2 for 16 h and control cells were treated with DMSO. Cells were harvested and subjected to caspase 3/7 activation assay by using caspase-3/7 assay kit. The caspase-3/7 activities relative to the untreated control were plotted for compounds 1 and 2 (1, H and 2, CH_3). Compound 1(1, H) was used as a positive control. Each treatment was done with 4 replicates. Error bars indicate standard error of the mean. This experiment was performed by Dr. Sahba Kasiri and the figure was made from his results.

3.3.4 Ascorbic acid inhibits apoptosis induced by Fe(III)-salphen derivatives

Ascorbic acid (vitamin C) is an antioxidant vitamin that plays an important role in cancer prevention by scavenging free radicals that can damage DNA (*54*). However, a high level of antioxidant is not favorable in some stages of cancer because it can cause resistance to apoptosis and prevent the removal of genetically damaged or mutated cells (*54*). Wenzel et. al. have shown that ascorbic acid inhibited camptothecin (inhibitor of DNA topoisomerase I) and flavone induced apoptosis in a dose-dependent manner by decreasing the levels of caspase-3 (*54*). Ascorbic acid was also shown to inhibit apoptosis by scavenging the free radicals and reactive nitrogen species in the mitochondria of cancer cells increasing the cancer cell population (*54*). In order to

examine inhibitory properties of ascorbic acid on apoptosis induced by Fe(III)-salphen derivatives, CCL228 cells were co-treated with increasing concentration of ascorbic acid in presence of 1 µM of compound 1 (1, H) or compound 2 (2, CH_3). The results showed that the apoptosis induced by Fe(III)-salphen derivatives was inhibited by ascorbic acid in a dose-dependent manner (figure 3-5) suggesting the involvement of oxidative stress and ROS in apoptosis (*55*). The effect of ascorbic acid on the cell viability was more pronounced in compound 1 than in compound 2. When compared to the control, there was up to 25 fold increase in the cell viability of compound 1 (1, H) treated CCL228 cells and up to 20 fold increase in the cell viability of compound 2 (2, CH_3) treated CCL228 cells after co-treatment with ascorbic acid (25 to 200 mM). These results suggest that both compounds 1 and 2 (1, H and 2, CH_3) mediate apoptosis by inducing oxidative stress in the cancer cells. The addition of ascorbic acid removes the ROS from the environment and prevents the cells from apoptosis. It is also indicative that cancer cells can develop resistance against compound 1 (1, H) more easily than compound 2 (2, CH_3) at high levels of antioxidants by inhibiting apoptosis (*54*).

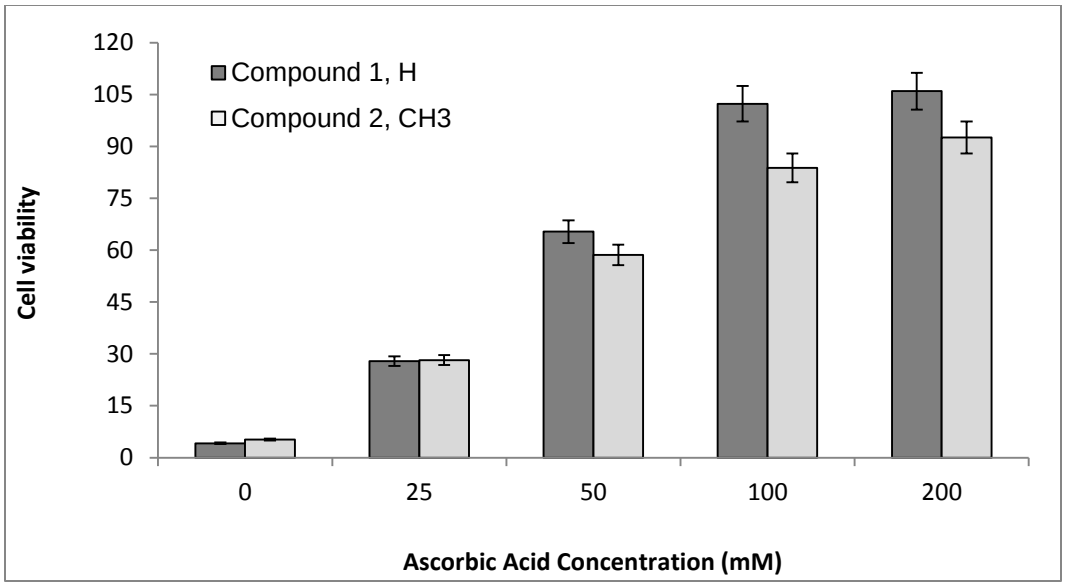


Figure 3-5 Ascorbic acid inhibits apoptosis induced by Fe(III)-salphen derivatives CCL228 cells were grown in 96 well plates until they were about 50% confluent and were treated with increasing concentration of ascorbic acid in presence of 1 µM of compound 1 (1, H) and compound 2 (2, CH_3) and the cell viability was measured by MTT assay after 96 h. The number of viable cells relative to control was plotted against concentration of ascorbic acid. The error bars indicate standard error of the mean.

3.3.5 The effect of Fe(III)-salphen derivatives on cell cycle

Anticancer drugs should be able to selectively target cancer cells by detecting the differences between cancer and normal cells (*56*). The overexpression of cyclins in tumor cells is one of the major differences (*56*). Cyclins are involved in eukaryotic cell cycle regulation and help in the cell cycle progression by activating their corresponding CDKs (Cyclin-dependent kinases). Eukaryotic cell cycle consists of four phases: S-phase (DNA replication), M-phase (mitotic phase), G1- and G2-phase (gap phase; cell prepares for S and M phase) (*57*). Cyclins (A, B, D, and E) form cyclin/CDK complex with respective CDKs and their expression levels is regulated in different phases of cell cycle (*57*). Cyclin D/CDK4, cyclin D/CDK6, and cyclin E/CDK2 control the progression from G1 to S phase of cell cycle (*58*). Likewise, cyclin A/CDK2 and cyclin B/CDK1 control the progression from S phase to M phase (*57*). Since cyclins control the progression of cell

cycle, their levels have to be much higher in tumor cells than in normal cells. For example, cyclin B1 (required for progression through mitosis) is overexpressed in several types of cancer including prostate cancer (*56*) and cyclin D1 is known to be overexpressed in breast cancer (*59*), liver cancer (*60*), head and neck cancer (*61*). Small molecule inhibitors of cyclins and CDKs are being investigated for their anti-proliferative effect in tumor cells and are targets for new anticancer drugs (*56*). For example, C-X-C motif chemokine ligand 14 has been shown to suppress tumor cell proliferation by down regulating the levels of cyclins and CDKs (*62*). Mukhopadhyay et. al. have shown that the a chemotherapeutic agent (curcumin) inhibits the proliferation in prostate and breast cancer cell lines by down-regulating cyclin D1 at mRNA and protein levels (*63*). Cyclin D1 is known to induce neuronal apoptosis without the involvement of tumor suppressor protein p53 (*64*). Gomez et. al. have shown that potential chemotherapeutic drugs (2-methoxyestradiol and paclitaxel) increase the level of cyclin B1/CDK2 and induce apoptosis in human cervical cancer cells by arresting the cells in G2-M phase (*56*), (*65*). Similarly, siRNA mediated knockdown of cyclin B1 levels has been shown to decrease apoptosis induced by 2-methoxyestradiol (*56*), (*66*). Moreover, Cisplatin is also known to induce apoptosis by arresting the cells in the G2-M phase of the cell cycle (*67*).

In order to investigate the effect of Fe(III)-salphen derivative on the cell cycle regulatory genes, CCL228 cells were treated with 1 µM of compound 2 (2, CH_3) or 15 µM of Cisplatin for 2 to 16 h after. The treated cells were analyzed for the expression of cyclins (A, B, D, and E) at mRNA levels by polymerase chain reaction (PCR) using gene specific primers listed in table 3-1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control to see if an equal amount of mRNA was used for PCR. GAPDH is a housekeeping gene that is assumed to be expressed equally in treated and untreated cells but the expression levels can vary between different cell lines (*68*).

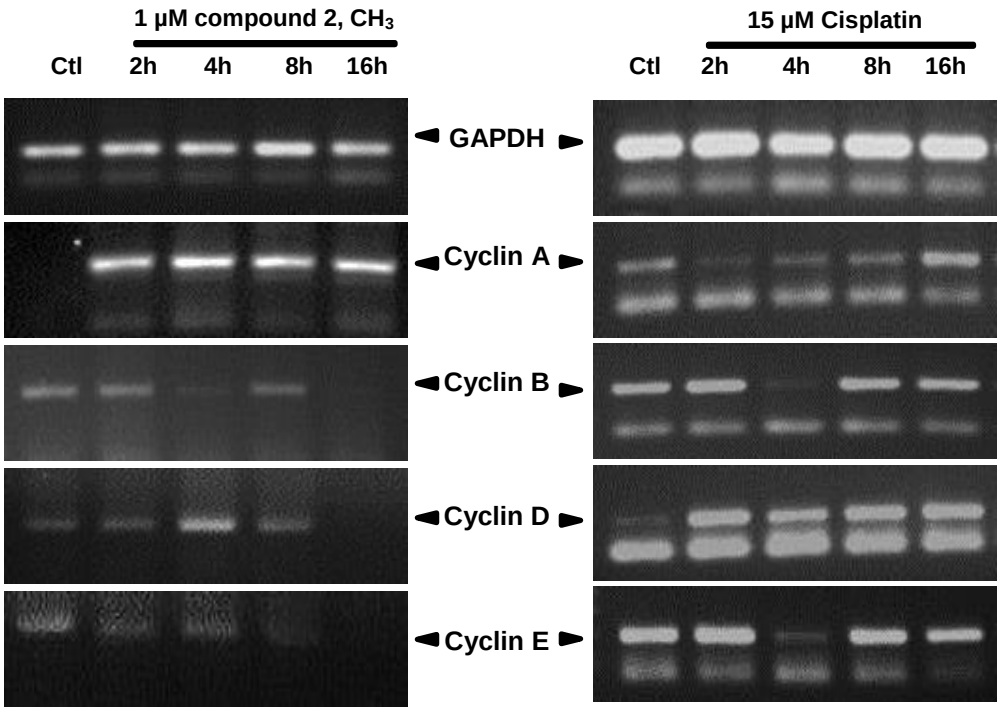


Figure 3-6 Effect of Fe(III)-salphen derivative on the expression of cell cycle genes.

Monolayer of CCL228 cells grown in 60 mm plates were treated with 1 μM compound 2 (2, CH_3) or with 15 μM Cisplatin and the control cells were treated with equal amount of DMSO. All the plates were incubated for 2, 4, 8, and 16 hours after which the cells were harvested and subjected to RNA extraction, cDNA synthesis, and PCR as described previously by Mandal lab (37). Specific primers were used to amplify the gene of interest. The PCR product was loaded onto 1.5% agarose gel and was subject to gel electrophoresis. The gel images were taken using Alpha imager. The top band corresponds to the primer band. Each experiment was repeated three times.

On treatment with 2, CH_3 the expression levels of cyclin A and cyclin B were not affected but that of cyclin D and E were slightly affected (figure 3-6, left). No product band for cyclin A is seen in lane 1 (untreated control) which could be due to PCR problem. Cyclin D level increased at 4 h as compared to the untreated control after which it decreased; and cyclin E levels decreased as compared to the untreated control (figure 3-6). The results of Cisplatin treatment showed that the expression of the cyclins increased at 16 h when compared to the untreated control (figure 3-6). The fluctuations in the

expression levels between time points could be due to unequal loading of the PCR product. For Cisplatin, 4 h treatment consistently gave low product as compared to 2 h and 8 h samples which could be due to PCR problem in that lane. The amplification of primer (top) and dimer (bottom) gave rise to the two bands that can be seen in most cases. These results suggest that Cisplatin induces cell proliferation by progressing through the cycle as indicated by the high cyclin levels. However, no conclusive remarks can be made about the effect of Fe(III)-salphen derivative (compound 2, CH_3) on the cell cycle genes. More work has to be done to specifically check the levels of each of the cyclins at different phases of cell cycle.

3.4 Summary and Conclusion

Large number of compounds are synthesized and screened every year as a potential drug but very few go into clinical trials and success rate is very low. Among them anticancer drugs are widely studied because the available ones have several side effects and are limited to certain types of cancer. The design of new drugs is of challenge because it is difficult to predict the physiochemical interactions of these complexes in the body which leads to several side effects. Although numerous attempts are made in the discovery of novel anticancer drugs there is always a necessity to synthesize new compounds and to study their biochemical activities in detail. Biochemical evaluation of these newly synthesized compounds is required to find the mode of action and pathway involved so that they can be modified to selectively target the cancer cells and to overcome the side effects. In this context, six different derivatives of Fe(III)-salphen (1, H) were biochemically evaluated to elucidate the possible mechanism involved in the cytotoxicity. These compounds induced apoptosis in CCL228 cells as characterized by condensation and fragmentation of the nucleus observed by DAPI staining. Compound 2

(2, CH_3) with lowest IC_{50} (~ 0.3 µM) and highest toxicity was further investigated for its biochemical activities. DAPI staining of the nucleus after treating CCL228 cells with compound 2 (2, CH_3) over a period of 72 hours showed that it induces apoptosis in a time dependent manner. Compound 2 (2, CH_3) also activated the executioner caspases-3/7 in CCL228 cells which further supports that apoptosis is involved in the toxicity. Router et. al. have postulated that Fe-salen generates hydroxyl radicals in cooperation with the quinine system that aids in the formation of iron(III) O^{2-} species (*69*), (*11*). Ascorbic acid assay was performed on CCL228 cells treated with 1 µM compounds 1 and 2 to investigate the involvement of free radicals. Ascorbic acid is an antioxidant that is known to scavenge free radicals and ROS from the body and protect the DNA from oxidative damage and apoptosis. The apoptosis induced by compounds 1 and 2 (1, H and 2, CH_3) was almost completely inhibited by ascorbic acid at higher concentrations. This result suggests that Fe(III)-salphen derivatives effectively generate free radicals and ROS that in turn mediate apoptosis. Since cyclins are known to be overexpressed in cancer cells, we analyzed the cyclins (A, B, D, and E) at mRNA levels after treatment of CCL228 cells with compound 2 (2, CH_3) and Cisplatin. No obvious relationship between the cytotoxicity and the cell cycle regulatory genes could be established but it appears that Cisplatin and compound 2 (2, CH_3) act differently on these cyclins. Further work has to be done to study cell cycle in detail by arresting the cells at different phases and analyzing the expression of cyclins at protein and mRNA levels. Moreover, antisense mediated knockdown of certain cyclins can be done to understand their roles in tumor proliferation and Fe(III)-salphen mediated apoptosis. Cytochrome c assay can be done to show the involvement of mitochondrial pathway of apoptosis.

Appendix A

IR Spectrum of Fe(III)-salphen derivatives

Transmittance [%]
10
20
30
40
50
60
70
80
90
Wavenumber cm-1
3500
3000
2500
2000
1500
1000
500
3043.67
1607.88
1529.95
1635.19
1462.74
1440.54
1373.97
1341.67
1316.76
1258.08
1196.80
1144.49
1130.56
1027.32
979.82
914.34
857.70
818.55
761.59
610.45
569.08
552.54
504.62
473.97
CH3
N
N
Fe
O
O
Cl

Single channel
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
3500
3000
2500
2000
1500
1000
500
3422.46
2940.75
2605.17
2497.83
1607.76
1582.79
1535.85
1502.49
1465.03
1444.30
1378.26
1313.55
1262.80
1189.91
1150.67
1128.11
1112.55
1034.66
967.11
913.25
821.26
758.57
606.06
531.79
496.69
474.39
OCH3
N
N
Fe
O
O
Cl
BRUKER

Transmittance [%]
20
40
60
80
100
120
140
Wavenumber cm-1
3500
3000
2500
2000
1500
1000
500
3063.81
2360.61
1632.84
1558.51
1497.41
1462.20
1441.10
1377.90
1261.65
1176.52
1144.39
1028.40
982.52
917.44
847.79
823.55
761.77
668.23
610.19
550.46
504.17
419.74
F
N
N
Fe
O
O
Cl
BRUKER

Transmittance [%]
10
20
30
40
50
60
70
80
90
Wavenumber cm-1
3500
3000
2500
2000
1500
1000
500
BRUKER
Cl
N
N
Fe
O
O
Cl
3068.00
1602.20
1575.10
1532.00
1461.67
1439.72
1374.94
1320.13
1253.90
1195.14
1145.78
935.19
904.34
859.84
816.02
761.10
604.41
548.65
497.75
475.54
433.45

Transmittance [%]
20
40
60
80
100
Wavenumber cm-1
3500
3000
2500
2000
1500
1000
500
3053.77
1602.10
1572.40
1532.66
1461.09
1438.90
1372.94
1313.19
1194.34
1149.99
1129.50
928.67
894.02
858.59
814.47
760.05
602.68
547.40
495.59
Br
N
N
Fe
O
O
Cl
BRUKER

Transmittance [%]
100
80
60
40
20
Wavenumber cm-1
3500
3000
2500
2000
1500
1000
500
3059.82
1658.80
1604.52
1577.08
1532.88
1462.03
1441.28
1374.92
1315.87
1272.93
1248.57
1196.32
1149.13
1129.47
919.16
871.24
814.49
760.38
712.16
662.30
622.69
550.46
493.45
BRUKER
O
C
N
N
Fe
O
O
Cl

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