ANTIOXIDANT AND ANTICANCER PROPERTIES OF METALLO-SALENS

By

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the requirements

for the Degree of

MASTER OF SCIENCE IN CHEMISTRY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2015

Acknowledgements

I would first like to thank Dr. Subhrangsu S. Mandal for the excellent mentorship and the support he has provided to me as an advisor. He is an excellent teacher and well versed in all areas of research. With his guidance I have learned more than I can learn from books. His instructions have helped me in gaining problem solving capability and critical thinking.

I would also like to give my special thanks to my committee members, Dr. Kayunta Johnson-Winters and Dr. Jongyun Heo. My heartful acknowledgements and thanks to my group members Arunoday Bhan, Paromita Deb, Koushik Raghavan and Nadine Z Shihabeddin for their teamwork, advice and helping me to understand some important concepts in research. I would also like to thank the Department of Chemistry and Biochemistry for providing me an opportunity to pursue my graduate study.

Final and special thanks to my family and friends for their endless love and support that has allowed me to achieve academic success.

April 24, 2015

Abstract

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Current anticancer agents do not greatly differentiate between normal cells and cancer cells. This leads to adverse effects and systemic toxicity, most of the drugs produce side effects in other tissues which limit the maximum allowable dose of drug. In an effort to find a novel metal complex that can be used in cancer therapy, a series of Mn(III) salen derivatives were synthesized and then characterized biochemically. A total of 18 compounds were tested on human neuroblastoma cells (SH-SY5Y) and immortalized mouse hippocampal cell line (HT22). Sixteen out of eighteen compounds showed cytotoxic effects towards human neuroblastoma cells (SH-SY5Y) at very low concentrations with IC50 values ranging from 1.5 to 76 μM. Fifteen out of eighteen compounds showed low cytotoxicity on HT22 cell line. The drugs were also screened in presence of ethanol which causes DNA damage and stops cells from repairing the damage. The drugs showed strong cytotoxicity towards SH-SY5Y in the presence of ethanol, surprisingly the same drugs showed least effects on the HT22 cells. In fact they rather promoted cell growth at higher concentrations. Nuclear fragmentation in the presence and absence of drug and ethanol was studied by staining cells with DAPI (4',6-diamino-2-phenylindole). Differential interference contrast (DIC) microscopy images were taken to check the cell morphology. These Mn(III) salen derivatives showed great specificity towards cancer cells and they are very effective at lower concentrations compared to most widely used anticancer drug Cisplatin.

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INTRODUCTION

1.1 Metal Complexes in Medicine

Metal ions play an important role in biology; they stabilize essential biological structures such as proteins and DNA. Additionally metal ions maintain osmotic balance, nerve function and play a vital role in all the living systems. Deficiency of these metal ions in body leads to conditions like anemia, infertility and may even cause death [1]. Most of the heavy metals like bismuth, gold, silver, ruthenium and platinum exceeding normal amounts can produce heavy metal poisoning.

Despite their toxic effects, metals and metal complexes have been successfully exploited for their medicinal usage. Some of the earliest examples date as far back as 3000 BC, where ancient Egyptians used copper sulfate to sterilize water used in tonics. Gold has been used in dentistry and zinc was reportedly used to promote the healing of wounds by the Romans. Mercury (I) chloride was traditionally used in the 16^{th} century as diuretic and laxative, by 19^{th} century HgCl was incorporated in a tonic known as blue mass [1]. Metal complexes began to be screened more systematically for their medicinal properties in early 20^{th} century. In 1909 arsphenamine (salvarsan) became the first modern chemotherapeutic agent for treatment of syphilis. Since then a wide range of compounds containing transition metals and main group elements has been studied for their anticancer properties [2]. The application of inorganic chemistry to medicine is rapidly developing and most of the major classes of pharmaceutical agents contain metal compounds which are in current clinical use. They have been used in treatment as anti-protozoal, antiulcer, antimicrobial, antiarthritic, antimalarial and anticancer agents [2]. Gold metal complexes including gold nanoparticles, gold Nano-spheres gained importance due to their anticancer properties. Transition metal complexes are valuable in

medicinal chemistry as they include metals that are naturally present at enzyme's active site. Oxidation state of the central complex determines the biological activity of the metal complex. 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]. They produce their effect by covalently binding to DNA so as to form adducts that interferes with transcription and DNA replication, thereby they trigger cell death [1]. They are also known to affect the cellular function by inhibiting enzymatic activities. They displace the native metals present at the active sites of enzymes and inhibit enzyme function [4]. The positively charged central ion allows the complex to interact with the negatively charged phosphate backbone of DNA. They also tend to bind to the negatively charged carboxyl and sulfhydryl groups of proteins [1]. 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

Cisplatin is the first member of platinum containing anticancer drugs. It was discovered in early 19^{th} century and was known as Peyrone's chloride [5]. Heavy metals which were previously associated with lethal diseases are now used to treat them. It was accidently discovered while studying the effect of electrical field on bacterial growth using platinum electrodes. Cisplatin (cis-$[Pt(NH_3)_2Cl_2]$) was first reported by Rosenberg in 1965 [4] and later on used to treat testicular cancer in 1978. It made about $160 million in sales by the end of 1989 [6]. Due to its effective anticancer properties, unique toxicity and that allows to be used in combination with other drugs made it successful. About 3000 products were synthesized later and screened for their anticancer properties. Among them Cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, nedaplatin, triplatin and

lipoplatin gained importance [7] (Figure 1-1). Picoplatin, nedaplatin and triplatin are still under clinical trials.

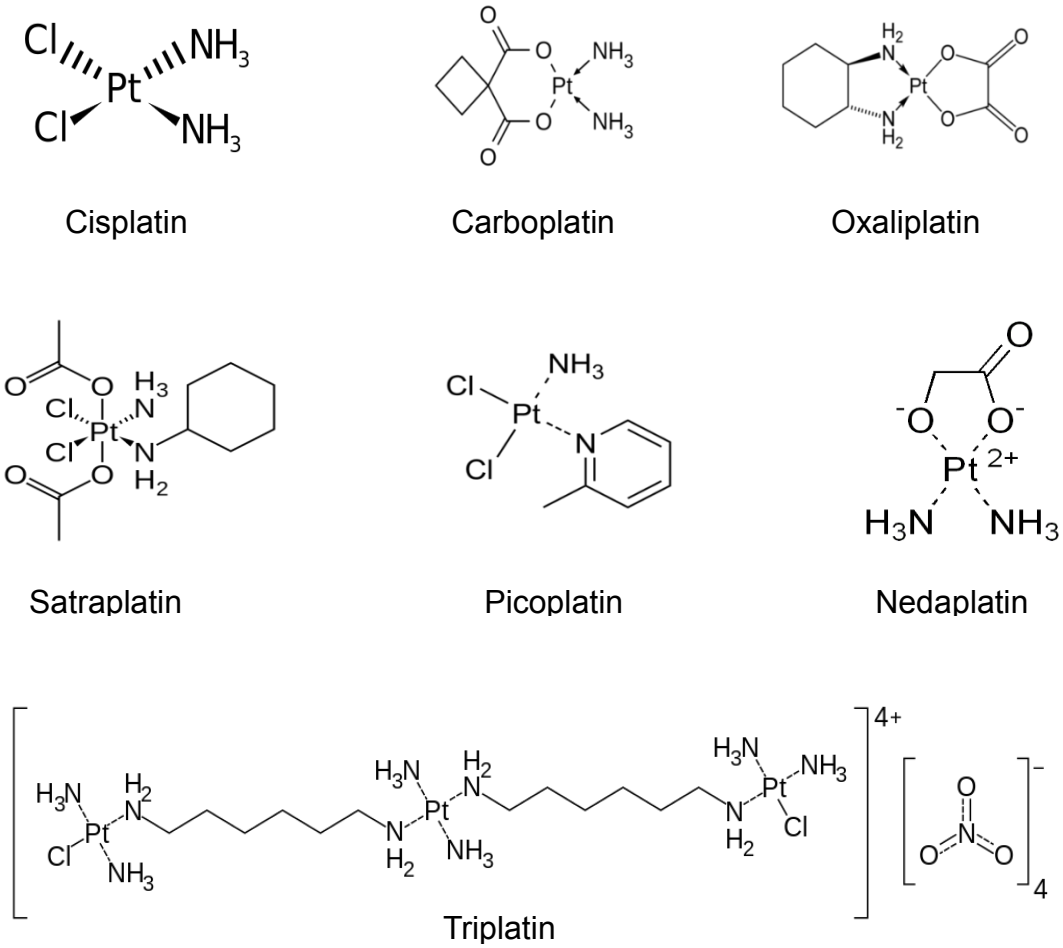
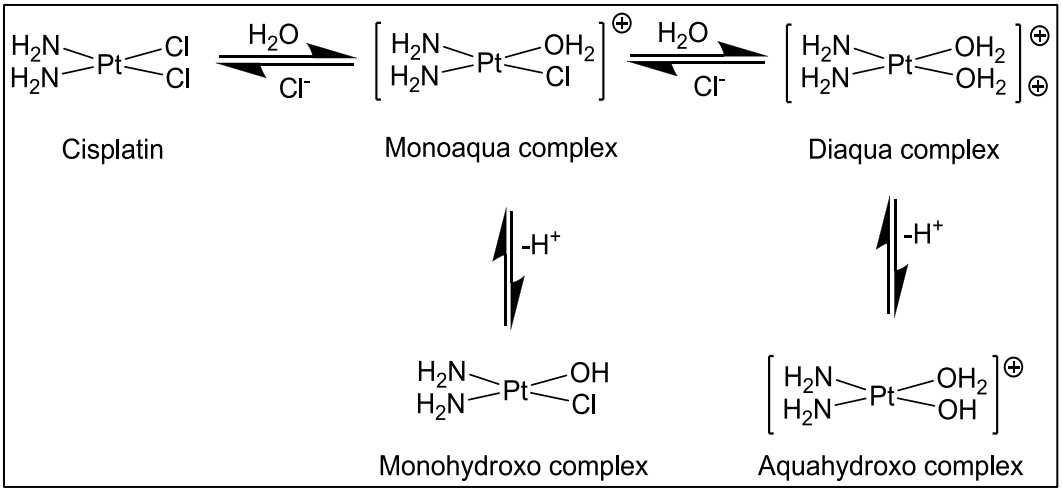


Figure 1-1 Structures of clinically used platinum based anticancer drugs. Redrawn from reference [8].

Cisplatin is absorbed by passive diffusion and gets hydrolyzed due to low chloride concentration within the cell (20 µM) [8]. Once inside the cell it hydrolyses to the active

diaqua species (scheme 1-1) [9]. This complex can irreversibly bind to two adjacent guanine bases (most electron rich sites) and crosslink to DNA [10]. The cross-linked DNA cannot replicate and the cell is forced to undergo apoptosis. Although cisplatin is most effective and successful drug, it has its limitations. For example multiple side effects arise due to its non-specificity which kill other cells and harm non-target tissues [11]. Cancer cells are prone to develop insensitivity to cisplatin because of decreased cellular uptake, decreased capability to crosslink DNA and increased cancer cell DNA repair [12]. Cisplatin cannot be used to treat all types of cancers as it does not show toxic response in them.



Scheme 1-1 Hydrolysis of Cisplatin to form biologically active platinum complex. Redrawn from reference [13].

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 the body. This causes nephrotoxicity [14]. Overuse of cisplatin and other platinum based antineoplastic agents can produce toxic effects such as ototoxicity, myleotoxicity, hemolytic anemia and neutropenia. Several experimental studies have been conducted

to control the toxicity of cisplatin. *Kaeidi et. al.,* showed that the toxic effects of cisplatin can be reduced by exposing cells to hyperoxic conditions. They also proposed that intitiating mild oxidative stress on cells before treatment can enhance defense mechanisms in cells to fight the reactive oxygen species generated by cisplatin [14]. There is always a demand for novel drugs specifically towards anticancer drugs with fewer side effects and a broad spectrum activity.

1.2.1 Non-Platinum Based Compounds As Potential Anticancer Agents

The limitations of platinum based antineoplastic agents towards cancer treatment have encouraged scientists to expand their research on non-platinum metal complexes with a hope of finding a novel drug with less severe side effects. They varied the central metal ion and the type of ligand used to produce new drugs. For example, cobalt, ruthenium, iron and gold based metal complexes have been evaluated in preclinical studies; and gallium, titanium and ruthenium based complexes are in phase I and phase II clinical trials. These metal complexes are being exploited in drug development because of their ligand binding affinities, oxidation states and coordination numbers [15]. Ferrocenium complexes were the first iron based compounds that were reported to have anticancer properties [16]. 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+}) gets oxidized to ferrocenium ions (Fe^{3+}) that can damage DNA by generating reactive oxygen species (ROS) [17]. Similarly, iron carbonyl nucleosides and Fe (II) complexes with pentadentate pyridyl ligands also exhibit anti-proliferative properties [18].

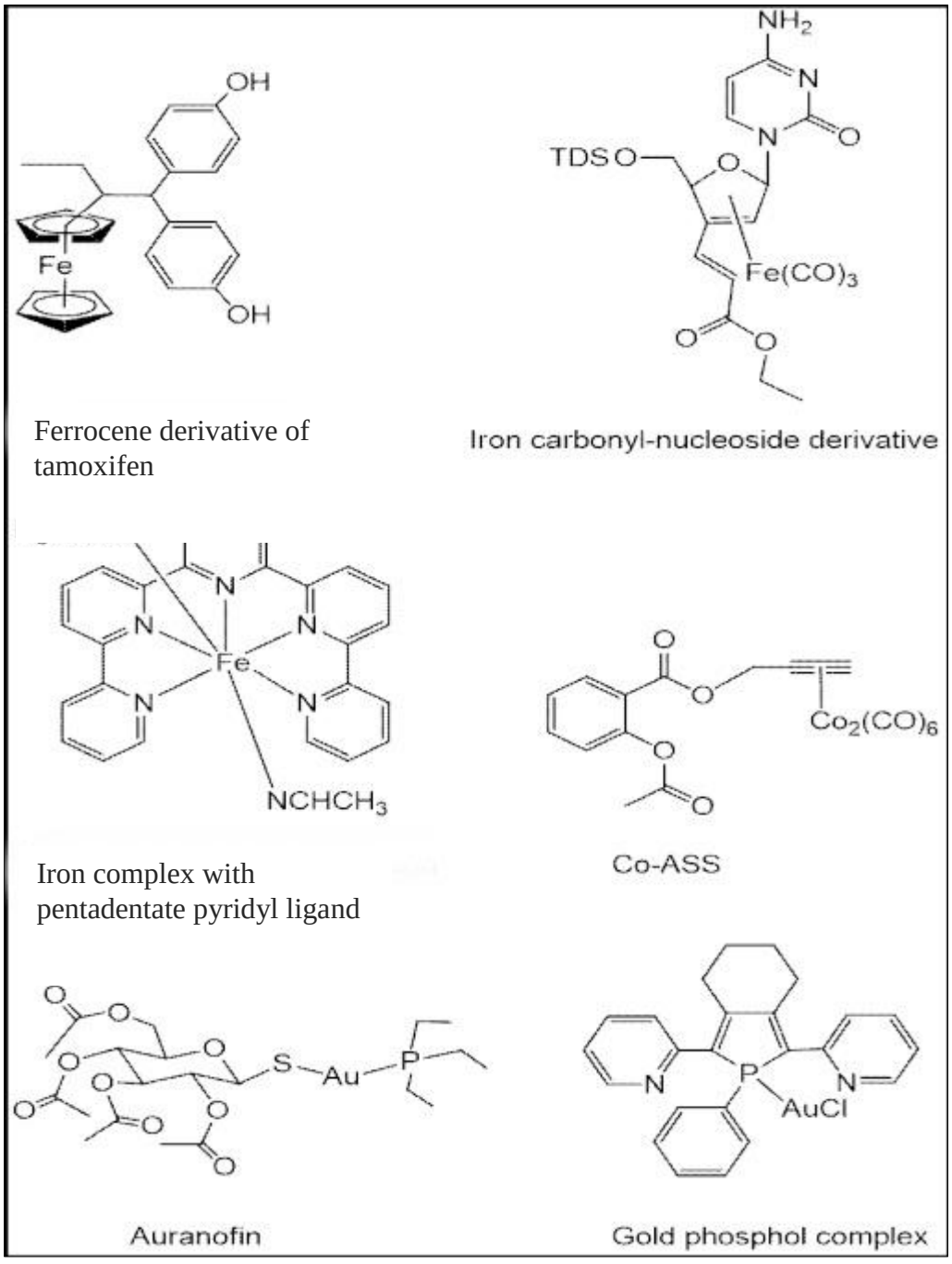


Figure 1-2 Structures of potential non-platinum anticancer agents. Taken from reference [16] and [19].

Cobalt is widely dispersed in the environment. Humans get exposed to it by breathing air, drinking water and eating food that contains cobalt. Skin contact with soil or water that contains cobalt may also enhance exposure. Recent years have seen a growing interest in the binding of small molecules to DNA and DNA cleaving with metal complexes. The interaction of transition metal complexes with DNA has been extensively studied in order to develop novel probes of DNA structure. Cobalt complexes have gained importance in that regard because they are potent hypoxia-activated prodrugs. For example, cobalt (III) complexes have the ability to cleave DNA under photolytic conditions [18]. Coordinated cobalt complexes are well known anti-inflammatory, antibacterial, and antifungal drugs [18]. For example, aspirin (a non-steroidal anti-inflammatory drug) becomes biologically active when coordinated to $Co_2(CO)_6$ (Co-ASS, Figure 1-2) [15, 20]. The excited state of cobalt (II) and cobalt (III) complexes brings about cleavage of macromolecules such as proteins and DNA [21]. Further, cobalt metal complexes with schiff base ligands has been shown to induce site-specific photocleavage of protein [22]. Targeted anticancer prodrugs present a therapeutic opportunity to deliver cytotoxins directly to a solid tumor while minimizing the toxic side effects of active drug exposure. Cobalt (III) chaperone complexes have been used for hypoxia selective delivery of a number of structurally different drugs, including the MMP inhibitor Marimastat (mmst), DNA minor groove alkylators and several nitrogen mustards, both *in vitro* and *in vivo*. Cobalt (III) complexes are highly inert and can form very stable complexes with cytotoxins, deactivating the cytotoxins by coordination and selectively releasing them in hypoxic tumor environments following reduction to cobalt (II) [23]. Gold complexes are also studied widely for their cytotoxic properties.

Gold (III) complexes are emerging as a new class of metal complexes with outstanding cytotoxic properties and are presently being evaluated as potential antitumor agents. This renewed interest is the result of recent studies in which various gold (III) complexes have been shown to be stable under physiological conditions. They also manifest relevant anti-proliferative properties against selected human tumor cell lines. Gold (III) complexes were chosen as a possible alternative to antitumor platinum drugs [19] as both platinum (II) and gold (III) complexes possess the same electronic configuration (d8) and give rise preferentially to square planar complexes. A very well-known gold complex called auranofin (Figure 1-2) was the first one found to exhibit anti-cancerous activity. They were found to inhibit the proliferation of human cervical cancer (*HeLa*) cells [19]. Several analogues of auranofin have also been shown to demonstrate anti-proliferative activity via inhibiting the activity of mitochondrial thiredoxin reductase; a selenoenzyme, which is known to reduce the antioxidant protein thioredoxin [19]. This is possibly driven by the exchange of gold ligands with selenocysteine and cysteine residues in the active site of thioredoxin reductase; thus, inhibiting the enzyme activity. Similarly, another gold complex (gold phosphol) (Figure 1-2) is also known to have cytotoxic effects on human glioblastoma cells by inhibiting the activity of glutathione reductase and thioredoxin reductase [19].

1.2.2 Non-Platinum Based Anticancer Drugs

1.2.2. A. Titanium based compounds

Rosenberg and group discovered the antitumoral properties of cisplatin [4, 25]. Several other platinum complexes such as carboplatin, oxaliplatin, nedaplatin, and lobaplatin are also popular antitumor agents [17]. However, it was found to be coupled with significant side effects like gastrointestinal and hematological toxicity. These led to the search for novel non platinum based antitumor drugs. Titanium complexes like

budotitane or the metallocene, titanocene dichloride are having been clinically tested for their cytotoxic activity in cancerous tissues [26, 27]. For example, budotitane was investigated in a clinical phase I trial and pharmacokinetic study administered as I. V. infusion twice weekly with a starting dose of 100 mg/m^2 [17].

1.2.2. B. Ruthenium based compounds

Ruthenium complexes were reported to possess anti-tumor potential in 1970 and 1980s [28, 29]. Later, ruthenium compounds were developed as anti-cancer agents and a lot of active agents have emerged as new potential drugs since that time. Some examples of Ruthenium complexes that enter clinical trials include Ru (III) complexes NAMI-A and KP1019 (FFC14a). KP1019 was found to exhibit better anti-proliferative effects on cultured cancer cells as compared to NAMI-A [30, 31]. However, NAMI-A type complexes were found to be significantly active in vivo due to the anti-metastatic properties of these agents. It is also shown to inhibit the expression of vascular endothelial growth factor (VEGF) involved in angiogenesis [32].

1.2.2. C. Gallium based compounds

Back in 1971, gallium salts were found to be antiproliferative [33, 34]. It was found to affect the structure, biosynthesis of DNA and proteins and was also found to be a potent inhibitor of enzymes like ATPases or DNA polymerases, and showed effects on mitochondrial function [35]. Several gallium compounds like gallium nitrate, gallium chloride, and gallium maltolate have been analyzed. For example, in a phase II study on metastatic urothelial carcinomas, gallium nitrate when applied intravenously in combination with vinblastine and ifosfamide was found to be effective against urothelial

carcinomas. Another active gallium nitrate complex mixed with vinblastine and ifosfamide was given to ovarian cancer patients [17, 36].

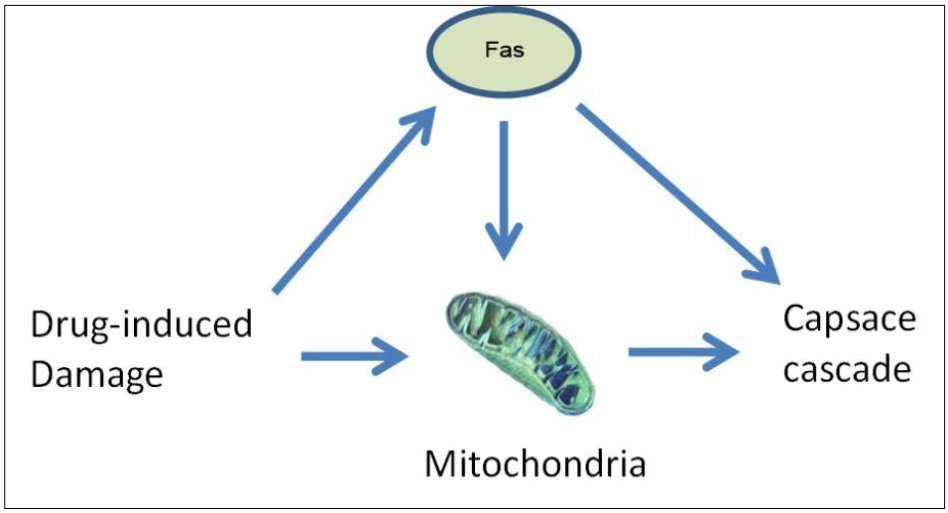


Figure 1-3 pathways involved in drug-induced cell death.

1.3 Cell Death Pathways as Targets for Anticancer Drugs

Apoptosis is a physiologic process that leads to cell death and eventually dead-cell clearance by neighboring phagocytes. Drug-induced damage to cell death involves two pathways –one is the mitochondrial release of pro-apoptotic molecules under the control of the Bcl- 2 family of proteins and the the other one is Death receptors of the tumor necrosis factor (TNF) receptor superfamily, namely Fas mediated apoptotic damage. Drug response may be modulated using these novel pathways of apoptosis (Figure 1-3).

1.3.1 The Mitochondrial Pathway

Anti-cancer agents can induce permeabilization of the mitochondrial membrane. In the inner membrane, it induces a dissipation of the proton gradient while in the outer membrane it induces the leakage of soluble proteins normally confined to the intermembrane space of these organelles. This pathway involves autonomous pore

formation in the external mitochondrial membrane by pro-apoptotic factors of the Bcl- 2 family such as Bax, which undergoes oligomerization by the truncated protein Bid (a BH3- domain-only protein of the Bcl-2 family).

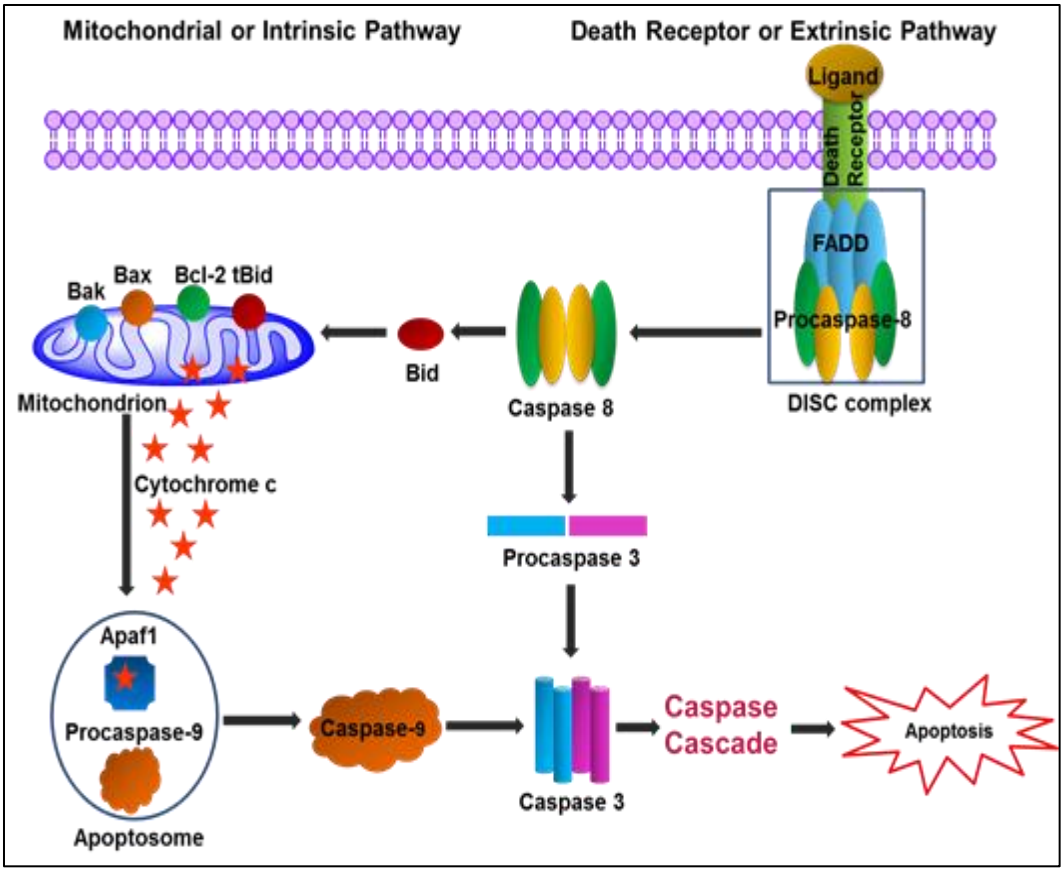


Figure 1-4 Pathways involved in apoptosis. Redrawn from reference [17].

This leads to the rupture of the outer-membrane of mitochondria [17]. Then a 14.5-kDa heme-binding holocytochrome-c is released which induces oligomerization of APAF1 (apoptotic protease activating factor 1), upon entering the cytoplasm, it exposes its caspase recruitment domain (ARD) in the presence of ATP [17]. Further, oligomerized APAF-1 binds to cytosolic procaspase-9 and forms an apoptosome complex and via

juxtaposition of two procaspase-9 molecules, leading to the release of mature caspase-9 (Figure 1-4). Pro-caspase 9 further activates downstream targets such as caspase- 3 and caspase-7 [17]. These processes may initiate certain catabolic reactions leading to cell damage [37].

1.3.2 The Death-Receptor-Dependent Pathway

The death receptor-dependent pathway induced apoptosis is also followed in some cell types in presence of a cytotoxic-drug. The death receptor Fas (also known as APO-1 or CD95) is 45-kDa protein present in type-1 membrane and is a member of the TNF receptor superfamily. The apoptotic cell death pathway triggered by its natural ligand. Fas ligand (FasL, APO-1L, CD95L FasL) associated cell death occurs in the following way: FasL binds to and induces clustering of Fas and leads to the formation of a complex known as the death-initiating signaling complex (DISC) which involves an adaptor protein named FADD (for Fas-associated death domain) [17] and procaspase-8 (Medema et al., 1997). FADD binds to the cytoplasmic region of Fas through its Death Domain (DD) and to the N-terminal domain of procaspase-8 via the death effector domain (DED). Oligomerization of procaspase-8 in the DISC results in the activation and release of caspase8.Depending on DISC level formed in each cell type, caspase-8 may either activate downstream effector caspases or cleave the proapoptotic member of the Bcl-2 family [17]. Translocation of the truncated Bid to the mitochondria activates, in combination with Bax, activates the mitochondrial pathway (Figure1-4).

1.4 Biological Applications of Metallo-Salens

Schiff bases are well-known drug candidates or diagnostic probes and analytical tools [38]. The Schiff bases are commonly used as anticancer including radioactive nuclide complexes, antibacterial, antifungal, antiviral agents [39, 40]. Schiff bases are also found in various natural, semi-synthetic, and synthetic compounds [41]. Several Schiff bases like the salen (N,N′-bis(salicylidene)-1,2-ethylenediamine) derivatives have become popular targets of cytotoxic drug study [42]. Metallo-salen complexes were initially used for olefin oxidation. Likewise, they are also now used to oxidize nucleic acids and hence lead to DNA or RNA damage [43, 44]. Asymmetric epoxidation of olefins was catalyzed by manganese (III) salen complex [45]. Hetero-Diels-Alder reaction and the Nozaki-Hiyama-Kishi reaction were efficiently catalyzed by chromium (III)-salen complex [46]. Several metallo-salen complexes have been found to have redox activities. As for example, manganese-salens (EUK-8 and EUK-134, Figure 1-5) are known to act like superoxide dismutase (SOD) and catalase which are well known reactive oxygen species scavengers [47-49]. These complexes are also well-known as reactive nitrogen species (RNS) scavengers indicating their diverse role [50]. Manganese salen complexes are very prone to oxidation in the presence of hydrogen peroxide, peracetate, persulfate, and peroxynitrate and readily form oxoMn-salen [48]. Oxidative as well as nitrosative-induced cell damage is controlled by oxo Manganese-salens via oxidation of NO to NO_2 and nitrite to nitrate [48]. Absence of such oxidation quenchers may lead to diseases like Alzheimer's disease, Parkinson's disease, motor neuron disease, and excitotoxic neural injury [50]. Cellular damage caused by Cd exposure is prevented by Mn-salen complexes via stopping the influx of Cd into cells eventually decreases ROS production in cells [50].

1.5 Metallo-Salen Complexes as Anticancer Agents

Metallo-salen complexes are classified as chemical nucleases that induce DNA-cleavage and apoptosis in cultured human cells [51, 52]. Metallo-salen complexes can simultaneously interact with DNA, initiate DNA cleavage, damage the DNA, and induce apoptosis in the target cells due to their flat electronic structure [53, 54]. For example, In vitro DNA cleavage has been shown to be induced by salen complexes of manganese, nickel, cobalt, ruthenium, iron, and copper [43]. Further, apoptosis and inhibition of cell proliferation is induced by cobalt salen (Figure 1-5) and the activity depends on the type of the bridging substituent [17]. Different forms of DNA cleavage is adopted by salen complexes of different metals. For example, Terminal oxidants help Manganese (III)-salen and Nickel (II)-salen complexes to cleave the DNA whereas Copper (II)-salen and Cobalt (II)-salen complexes make use of a reducing co-factor to initiate DNA cleavage[44]. Copper salen complex possess a unique hydroquinone system for DNA cleavage via unison with the copper redox system and simultaneous formation of oxidizing Copper (III) species [44]. Copper (II)-salen complexes have also been shown to bind to the grooves of DNA double helix and induce single-stranded breaks in the presence of a reducing agent [44]. Iron and copper salen complexes cleave DNA by forming hydroxyl radicals in presence of reductant, dithiothreitol whereas nickel salen complexes crosslink the DNA in the presence of oxidizers. Furthermore, cobalt salen complexes activate oxygen, naturally present in aqueous solutions to cleave DNA as compared to manganese salen complexes which damage DNA by their redox activities [51, 55]. Reducing agents such as glutathione help these compounds to damage DNA [56]. Our laboratory has previously shown that manganese (III) -salen and -salphen complexes are antiproliferative as well as apoptic in MCF7 cells which follows the mitochondrial pathway via nuclear pore formation and release of cytochrome c from the

mitochondria [57]. They have also demonstrated the selective cytotoxic nature of salen and salphen complexes of Fe (III) and Manganese (III) towards cancer cells over normal cells, which work in favor of anticancer drugs [57]. These observations suggest that these metallo-salen complexes (Figure 1-6) are novel anti-proliferative drug molecules for cancer therapeutics; however a thorough study is required for the complete analysis of their biochemical significance.

1.6 Effects of Alcohol on the Body

Ethanol (CH_3CH_2OH) is commonly called as drinking alcohol. It is volatile with a strong odor and is highly flammable. It is the principal type of alcohol found in spirits and alcoholic beverages. It's one of the recreational drugs which can produce neurotoxic effects [13]. When alcohol is consumed it is absorbed into blood stream and affects every part of the body. Depending on how much you consume and the physical condition of the patient, it has various short term and long term effects. Short term effects include drowsiness, vomiting, headaches, breathing difficulties, distorted vision, unconsciousness, anemia and coma [58]. Long term effects include alcohol poisoning, nerve damage, liver disease, high blood pressure, atherosclerosis, emphysema, vitamin deficiency stroke and sexual problems [59]**.** Reactive oxygen species (ROS) are small, highly reactive, oxygen containing molecules. They are naturally generated in small amounts in the body during metabolic reactions and are capable of damaging complex cellular molecules like DNA and proteins [60].

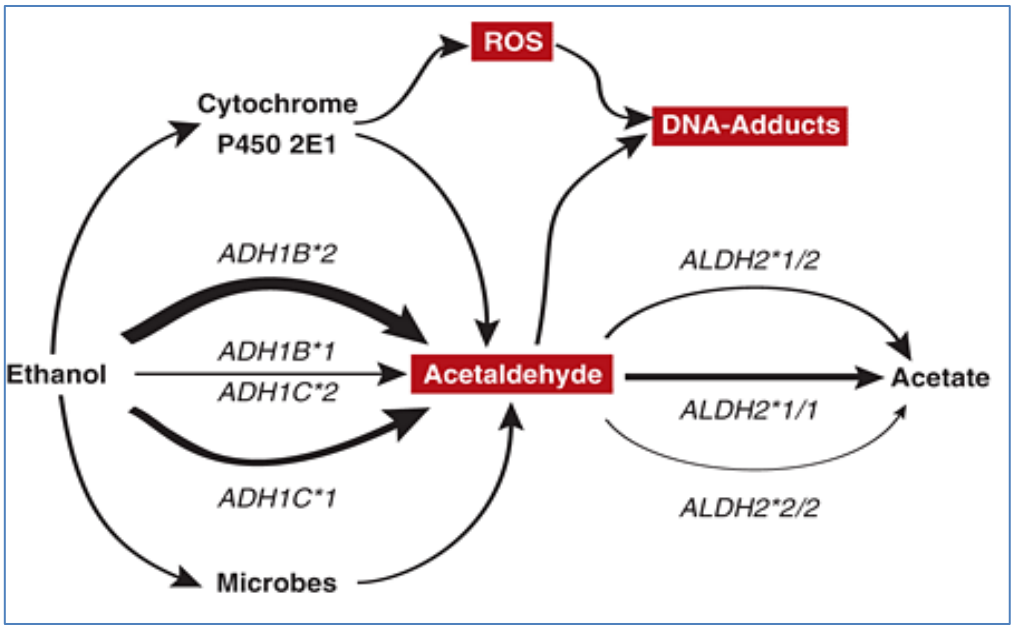


Figure 1-5 metabolism of alcohol and generation of ROS. Redrawn from reference [60]

Small amount of oxygen is converted to ROS in mitochondrial respiratory chain. Main source of ROS is the liver; it has numerous cellular systems which produce ROS. A group of enzymes called cytochrome P450 mixed-function oxidases are important in metabolizing various drugs, in this process it uses molecular oxygen and generates small amounts of ROS [61]. ROS is also produced by a variety of oxidation enzymes in the cells such as xanthine oxidase. Xanthine oxidase removes hydrogen from hypoxanthine and attaches it to NAD to produce NADH.

Due to disruptions in blood flow or alcohol consumption xanthine oxidase can convert into ROS producing oxidase, thereby enhancing oxidative stress on the cells. Immune cells like macrophages and neutrophils that help body from invading microorganisms can produce ROS, but it plays an important role in killing the microorganisms. Besides the natural production of ROS in the body; UV light, tobacco smoke and alcohol consumption can provide more oxidative stress. Medications taken are modified in the body by cellular enzymes to unstable intermediate; which react with molecular oxygen to produce the original drug and superoxide radical. Alcohol consumed is metabolized to acetaldehyde in the presence of alcohol dehydrogenase by using a molecule of NAD+. Acetaldehyde is further metabolized to acetate in presence of several alcohol dehydrogenases (Figure 1-5) [62, 63] shows the metabolism of ethanol and production of various free radicals. As ROS is naturally produced during various metabolic pathways, cells have developed several protection mechanisms against them. They deploy antioxidants on ROS and detoxify them. Under chronic or acute exposure to alcohol, ROS production is enhanced and causes disturbance in balance between the ROS production and ROS removal and repair of damaged complex molecules. Its effects include damage mitochondria resulting in increased ATP production [62]. Can produce alcohol-induced oxygen deficiency in liver lobules and also alter production of signaling molecules like cytokines causing effects on immune system [62, 63]. Alcohol also increase activity of the enzyme cytochrome P450 2E1 (CYP2E1), which metabolizes oxygen and can promote ROS generation. It also increases levels of iron in the cell which can promote ROS generation [61]. Acetaldehyde generated in ethanol metabolism was shown to promote cancer by interfering with several mechanisms, with replication of DNA. It can cause point mutations to gross chromosomal alterations. It affects the DNA repair mechanism and can interact with DNA building blocks to form

new molecules. These adducts may trigger replication errors and mutations in cancer causing genes. Cells in gastro intestinal mucosa may react directly with acetaldehyde to form crotonaldehyde, which can bind to DNA and cause mutations. It is capable of producing cancers in mouth and upper throat, larynx, oesophagus, breast, liver and bowel region [60, 61].

1.7 Metallo –salen complexes as antioxidant agents

Drugs with antioxidant properties are being widely proposed against several pathogenic disorders associated with oxidative damage caused by ROS (superoxide anion, hydroxyl radical and hydrogen peroxide). Metal compounds are well studied in drug research because they exhibit multiple oxidation states and can form stable complexes by coordinating with various ligands [64]. Recent studies show that metal complexes with Schiff base ligands could potentially facilitate the scavenging of ROS. This can restore the redox balance in damaged cells and organs. Schiff's bases are straight forward to prepare and versatile. A well designed Schiff's base is considered as privileged ligands, they stabilize different metal ions in various oxidation states controlling the performance of metals in various clinical applications. Schiff's bases are largely studied because of their ability to bind reversibly with oxygen redox systems and oxidation of DNA. The C=N link is essential for bioactivity and its antioxidant properties [65]. These complexes closely mimic active site of antioxidant enzymes and the redox property of a metal ion in the complex is critical in their activity. The Mn (II) complexes of pentaaza macrocyclic ligands are most thoroughly investigated molecules in an *in vivo* model. Most of these compounds showed antiarthritis, antitumor and pain-relieving properties [22, 38, 62] (Figure 1-6). The molecular mechanism is not well understood, however different rate determining steps for superoxide dismutation have been proposed based on stopped flow or pulse radiolysis experiments.

Aromatic and substituted ethylenediamine complexes have been reported to have antioxidant properties. Mn(III) complexes have been shown to scavenge O_2^-, H_2O_2 and lipid peroxides. In these salens manganese is coordinated to oxygen and nitrogen results in the formation of several possible valence states and these valence states play an important role in scavenging ROS [65].

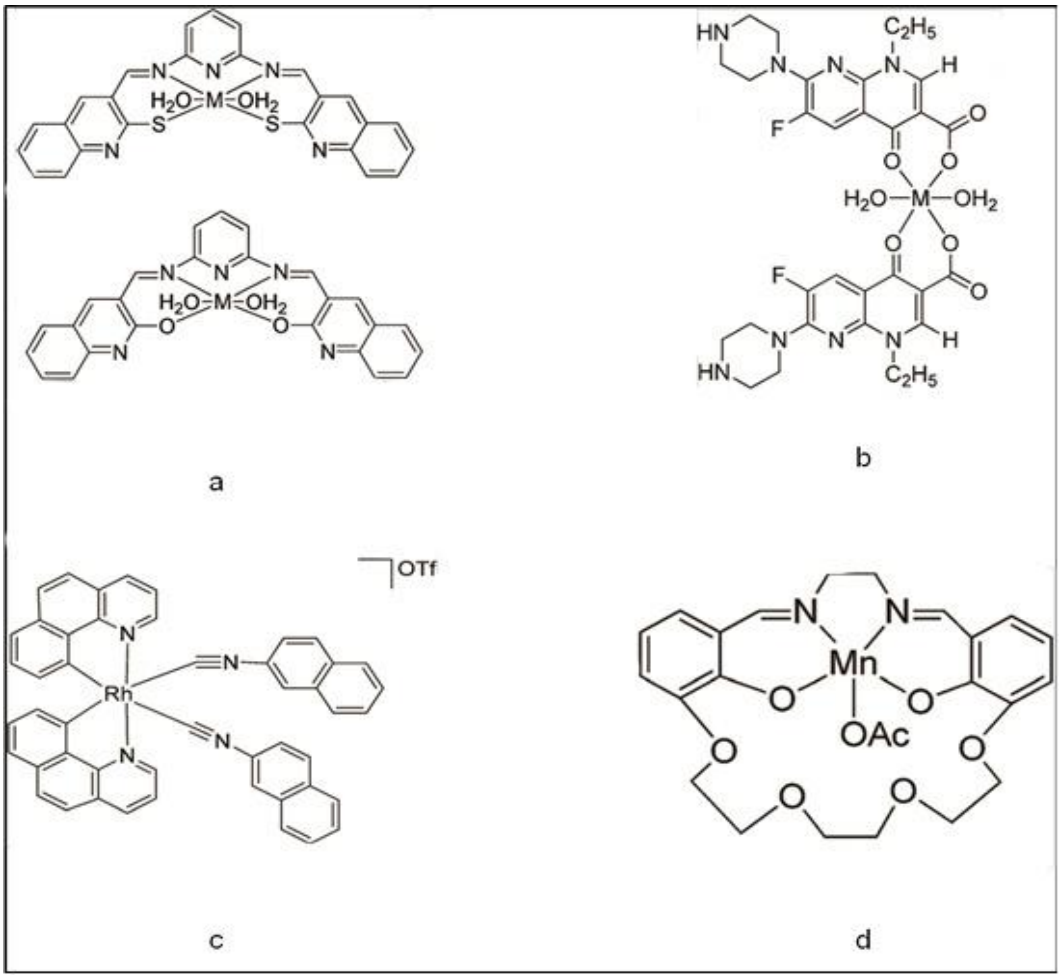


Figure 1-6 Structures of Metal complexes studied for their antioxidant properties-a)2-mercapto-3-formyl quionline derivatives, b) enoxacin derivative, c) cyclomethylated rhodium complex, d) Src homology 2 (SH2) phosphopeptide-binding domain. Structures redrawn from reference [65].

Chapter 2

SYNTHESIS OF NOVEL METALLO-SALEN DERIVATIVES AND EVALUATION OF THEIR ANTI-TUMOR ACTIVITIES

2.1 Introduction

There is a need for a novel anticancer drug which has a broad spectrum activity, minimal sideeffects and can overcome Cisplatin resistance. Drug should have high specificity towards tumor cell and the molecule should be relatively small. Small molecules reach target tissues quickly and doesn't need any artificial transport system. Metallo-salen compounds are a class of small molecule based drugs that have been studied for their antioxidant and anticancer properties [51]. Metallo-salens are straight forward to prepare and have versatile redox activities. Various metallo-salen complexes have been synthesized and tested for their anticancer activities [51,55]. Metallo-salens bind and cleave DNA [54]. Metallo-salens induced cell death is mediated by oxidative stress. There is a large potential of developing more metallo-salen derivatives with known mode of action and less adverse effects.

In this context I have studied the cytotoxicity of a total of 18 different derivatives of Manganese (III)-salen that possess varying the substituents on ortho, meta, para positions of the aromatic ring. These compounds were previously synthesized and characterized in Dr. Mandal's laboratory. These metallo-salens were then characterized by infrared (IR) spectroscopy. Elemental analysis was done before investigating their biological properties [57]. Cytotoxic studies of these compounds were performed and the IC_{50} values were compared to that of Cisplatin.

2.2 Materials and Methods

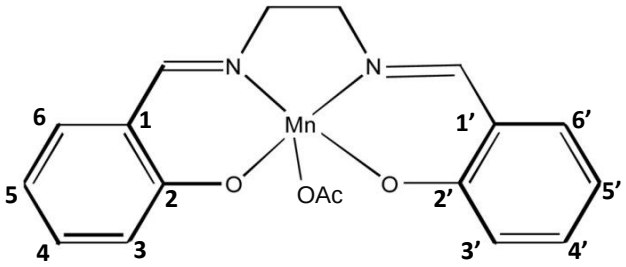
2.2.1 General

All reagents for organic synthesis and buffers were purchased from Sigma-Aldrich unless otherwise noticed. Tissue culture medium DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), penicillin and streptomycin were purchased from Sigma–Aldrich. 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. SH-SY5Y and HT22 were obtained from ATCC (American Type Culture Collection). Anhydrous Manganese acetate was purchased from Spectrum Chemical Manufacturing Corporation, DAPI (4′, 6-diamidino-2-phenylindole) was purchased from Invitrogen. 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 Manganese (III)-salen and their derivatives

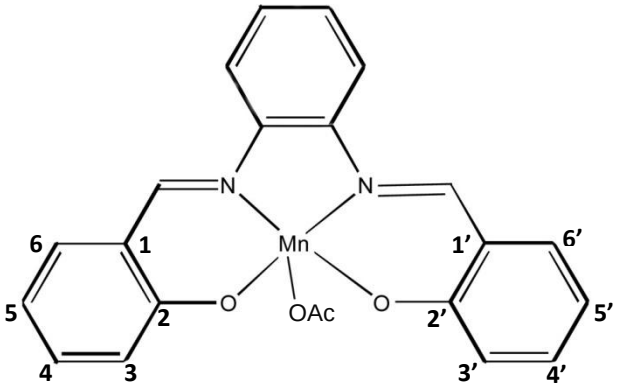
Manganese (III)-salen derivatives were synthesized and characterized by a common procedure that was previously described by Dr. Mandal lab. Manganese salens were synthesized by mixing with one equivalent of ethylenediamine with two equivalents of salicylaldehyde (or its derivatives) by stirring it over 60° C water bath for 15 min and then it is left aside overnight to let it cool down and precipitate out the metal-complexes. This produced a yellow to orange colored precipitate of respective salen derivatives. Precipitate was then filtered and washed using ice cold methanol. To prepare Manganese (III) salen derivatives, respective ligand derivatives were dissolved in methanol and mixed with equivalent amounts of anhydrous Manganese (III) acetate, heated to 60º C and stirred for 30 minutes. This resulted in a dark black/ brown solution

that was cooled to room temperature. The products were cleaned and characterized by ^{1}H and ^{13}C NMR and results were consistent with the expected products. The products were also characterized by mass spectroscopy (ESI-MS), infrared (IR) spectroscopy and elemental analysis [51, 52, 55, 57]. The observed values of C, H, and N composition of all the compounds were comparable to the calculated values. Results showed presence of water in these compounds. Compounds were dissolved in cell culture grade DMSO and used for cytotoxic assays. Mn(III) N,N'-bis (salicylidene) ethylenediamine(salen), Mn(III) N,N' bis(salicylidene)orthophenylene-diamine (salphen) and Mn(III) N,N'-bis(salicylidene) -2,3-naphthalenediamine (salnaphen) were synthesized as described above by previous members of Dr.Mandal's lab. (Compounds are shown in Figures 2-1, 2-2, 2-3 and 2-4). (Mandal, *et. al.,* (2012). U.S.Patent No.8198322. Austin, TX: U.S. Patent and Trademark Office).



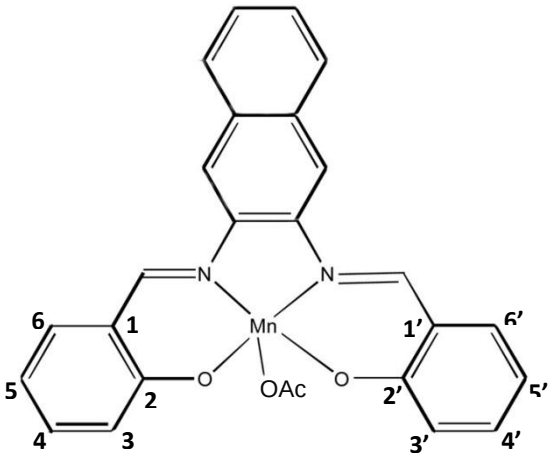
3, 3'= OH compound 1
4, 4'= OH compound 2
5, 5'= OH compound 3
3, 3'= OCH_3 compound 4
4, 4'= OCH_3 compound 5
5, 5'= OCH_3 compound 6

Figure 2-1 General structure of Mn(III)-salen derivatives (compounds 1-7).



3, 3'= OH compound 7
4, 4'= OH compound 8
5, 5'= OH compound 9
3, 3'= OCH_3 compound 10
4, 4'= OCH_3 compound 11
5, 5'= OCH_3 compound 12

Figure 2-2 General structure of Mn(III)-salphen derivatives (compounds 7-12)



3, 3'= OH compound 13
4, 4'= OH compound 14
5, 5'= OH compound 15
3, 3'= OCH_3 compound 16
4, 4'= OCH_3 compound 17
5, 5'= OCH_3 compound 18

Figure 2-3 General structure of Mn(III)-salen derivatives (compounds 13-18)

2.3 Cell Culture

Monolayer of human neuroblastoma cells (SH-SY5Y) and immortalized mouse hippocampal cell line (HT22) cells were grown and maintained in DMEM supplemented with heat inactivated FBS (10%), L-glutamine (1%) and penicillin/streptomycin (1%). Cells were grown in a humidity controlled incubator at 37ºC. Cells for cell viability and cytotoxicity were grown in 96 well micro titer plates. Cells for microscopy experiments were grown in 6 wells plates with coverslip in them.

2.4 MTT Assay and IC_{50} Determination

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed to determine the cytotoxicity of Mn (III) salen and its derivatives. Approximately 10,000 cells were seeded into 96 well micro titer plates (180 µL DMEM) and were grown to 50-70% confluence. The cells were then treated with 20 µL of compounds 1-18 dissolved in DMSO, the final concentration in the wells were maintained between 0 to 100 µM for 48-72 hours. Control cells were treated with same amount of DMSO and 180 µL DMEM mixed with 20 µL of DMSO was used as blank. Each reaction was performed in 4 wells in parallel. MTT assay was performed after 72 hours of treatment. 20 µL MTT (stock 5 mg/mL in PBS) was added into each well of micro titer plate and incubated for 2-3 hours. NAD(P)H-dependent cellular oxidoreductase enzymes in the viable cells convert MTT to formazan crystals. Media is then discarded and the formed formazan crystals were dissolved in DMSO and incubated for 2 more hours with continuous shaking. The absorbance is then measured at 560nm using a micro plate reader (Fluostar-omega, BMG Labtech). Percentage viable cells relative to control were calculated using the absorbance values. The percent viable cells were plotted as a

function of concentration to obtain the IC_{50} values. Each experiment was repeated twice to confirm the results.

2.5 Results

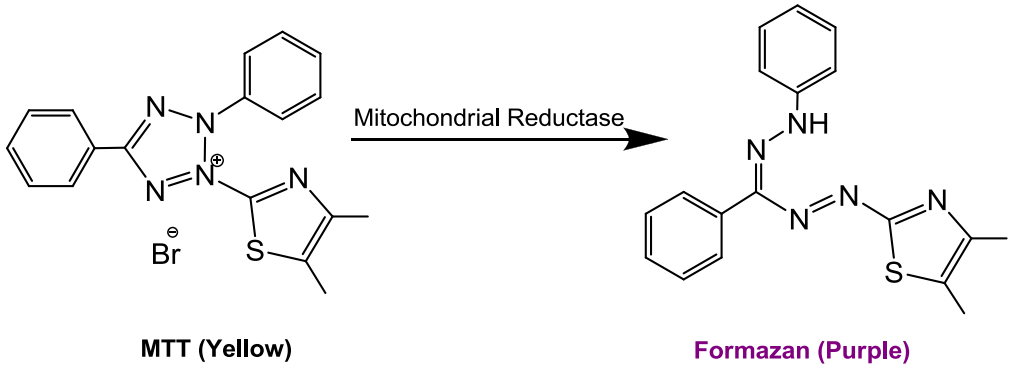
A total of 18 compounds were synthesized by altering the hydroxy, methoxy groups on aromatic rings and adding aromatic rings on the ethylenediamine bridge (Figure 2-1, 2-2, 2-3, 2-4). All compounds were evaluated for their cytotoxicity and biological significance.

2.5.1 Analysis of cytotoxicity of Manganese (III)- salen derivatives towards human cancer and normal cells

A total of 18 compounds were evaluated for their cytotoxicity in neuroblastoma (SH-SY5Y) and immortalized mouse hippocampal (HT22) cells. Cisplatin was used as the positive control. The cytotoxicity of these compounds in human breast cancer cells (MCF7) and normal breast epithelial cells (MCF10) have been analyzed previously [57] and compared in table 2-1.

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed to determine the cytotoxicity of Manganese (III) salen and its derivatives. Approximately 10,000 cells were seeded into 96 well micro titer plates (180 µL DMEM) and were grown to 50-70% confluence. The cells were then treated with 20 µL of compounds 1-18 dissolved in DMSO, the final concentration in the wells were maintained between 0 to100 µM for 48-72 hours. Control cells were treated with same amount of DMSO and 180 µL DMEM mixed with 20 µL of DMSO was used as blank. Each reaction was performed in 4 wells in parallel. MTT assay was performed after 72 hours of treatment. 20 µL MTT (stock 5 mg/mL in PBS) was added into each well of micro titer

plate and incubated for 2-3 hours. NAD(P)H-dependent cellular oxido-reductase enzymes in the viable cells convert MTT to formazan crystals (Scheme 2-1). Media is then discarded and the formed formazan crystals were dissolved in DMSO and incubated for 2 more hours with continuous shaking.



Scheme 2-1 Conversion of MTT to formazan in mitochondria of live cells [55].

The absorbance is then measured at 560nm using a micro plate reader (Fluostar-omega, BMG Labtech).

Table 2-1 IC_{50} values of Mn (III) salen derivatives towards cancer and normal healthy cell lines. A) MCF7 and MCF10 data was taken from reference[57]).

Compounds	IC50 (μM)			
	MCF7 (A)	MCF10 (A)	SH-SY5Y	HT22
3,3' dihydroxy Mn (III)-salen	36 ± 4.7	78 ± 3.2	23.1 ± 1.2	> 100
4,4' dihydroxy Mn (III)-salen	> 100	> 100	> 100	> 100
5,5' dihydroxy Mn (III)-salen	43 ± 3.7	73 ± 4.8	38.6 ± 0.8	> 100
3,3' dimethoxy Mn (III)-salen	17 ± 0.2	18 ± 1.3	3.45 + 1.1	> 100
4,4' dimethoxy Mn (III)-salen	12 ± 1.1	25 ± 2.9	2.45 +0.7	42.5 ± 0.7
5,5' dimethoxy Mn (III)-salen	16 ± 1.1	29 ± 2.9	52.5 ± 1.2	> 100
3,3' dihydroxy Mn (III)-salphen	17 ± 0.4	52 ± 0.5	2.6 ± 0.8	> 100
4,4' dihydroxy Mn (III)-salphen	> 100	> 100	> 100	> 100
5,5' dihydroxy Mn (III)-salphen	45 ± 3.6	48 ± 4.3	35.5 ± 1.5	> 100
3,3' dimethoxy Mn (III)-salphen	22 ± 0.8	28 ± 3.5	63.6 ± 1.9	> 100
4,4' dimethoxy Mn (III)-salphen	15 ± 0.2	32 ± 0.7	9.8 + 1.6	> 100
5,5' dimethoxy Mn (III)-salphen	55 ± 2.4	70 ± 7.0	> 100	> 100
3,3' dihydroxy Mn (III)-salnaphen	40 ± 1.5	127 ± 12	28.5 ± 1.2	> 100
4,4' dihydroxy Mn (III)-salnaphen	21 ± 0.7	102 ± 6.5	55 ± 0.8	> 100
5,5' dihydroxy Mn (III)-salnaphen	> 100	> 100	> 100	21 ± 2.1
3,3' dimethoxy Mn (III)-salnaphen	40 ± 1.5	61 ± 2.9	32.8 ± 0.9	8.5 +1.9
4,4' dimethoxy Mn (III)-salnaphen	20 ± 2	27 ± 1.2	60.7 ± 1.8	83 ± 2.5
5,5' dimethoxy Mn (III)-salnaphen	19 ± 1	101 ± 5.3	1.8 ± 0.7	48 ± 1.5

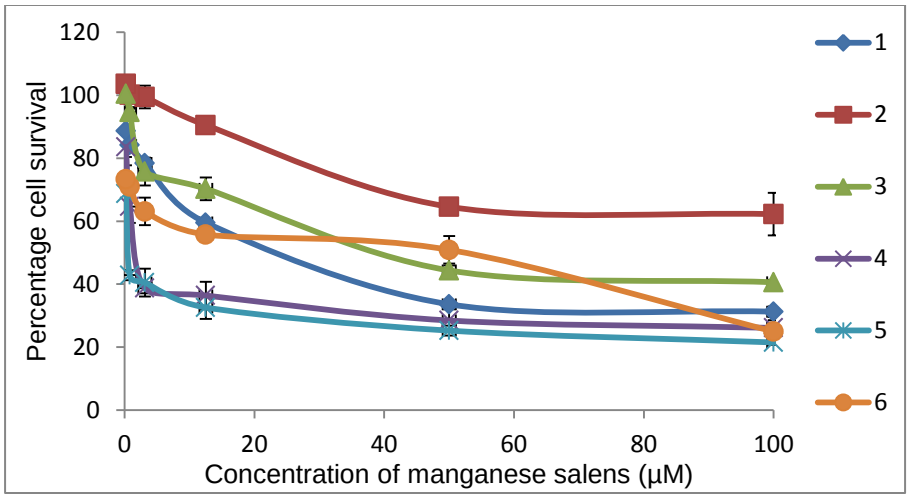


Figure 2-4 IC_{50} of Manganese (III) salens (compounds 1-6) on SY-SY5Y cell line.

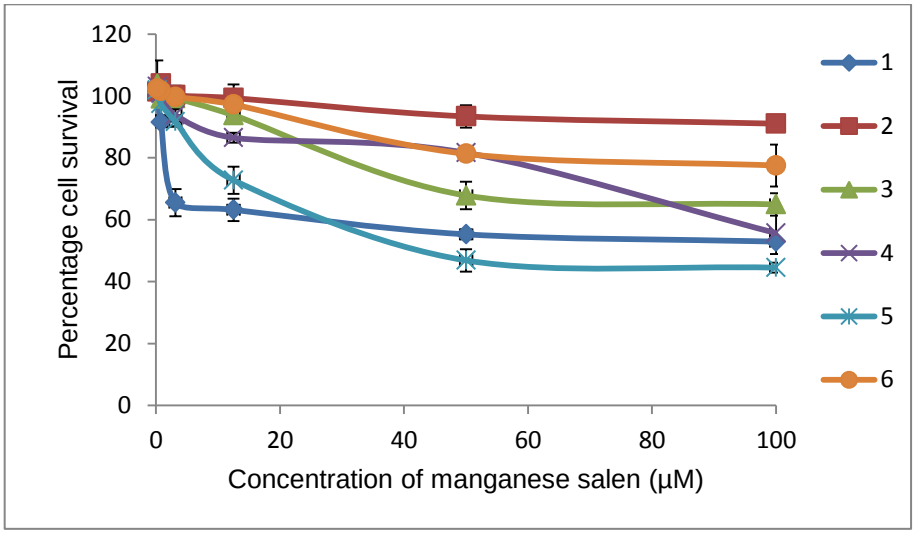


Figure 2-5 IC_{50} of Manganese (III) salens (compounds 1-6) on HT22 cell line.

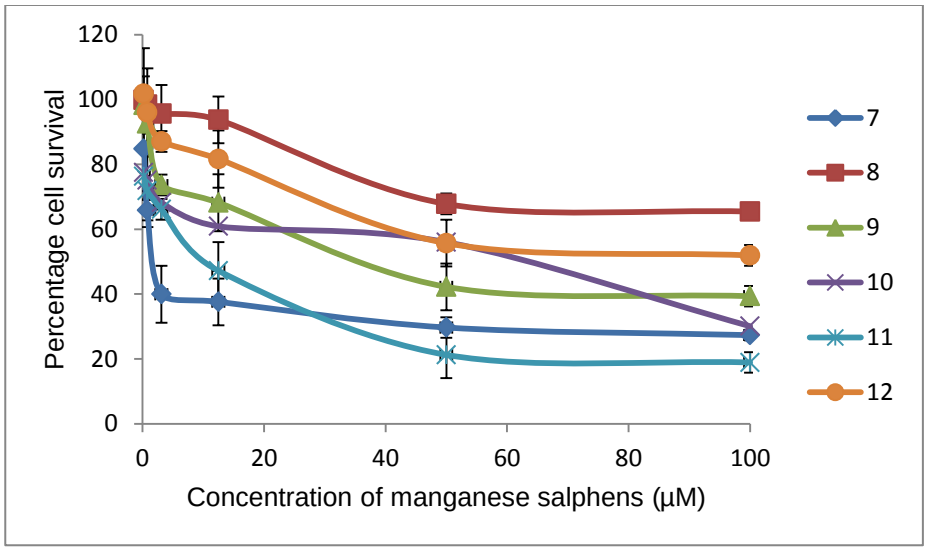


Figure 2-6 IC_{50} of Manganese (III) salphens (compounds 7-12) on SY-SY5Y cell line.

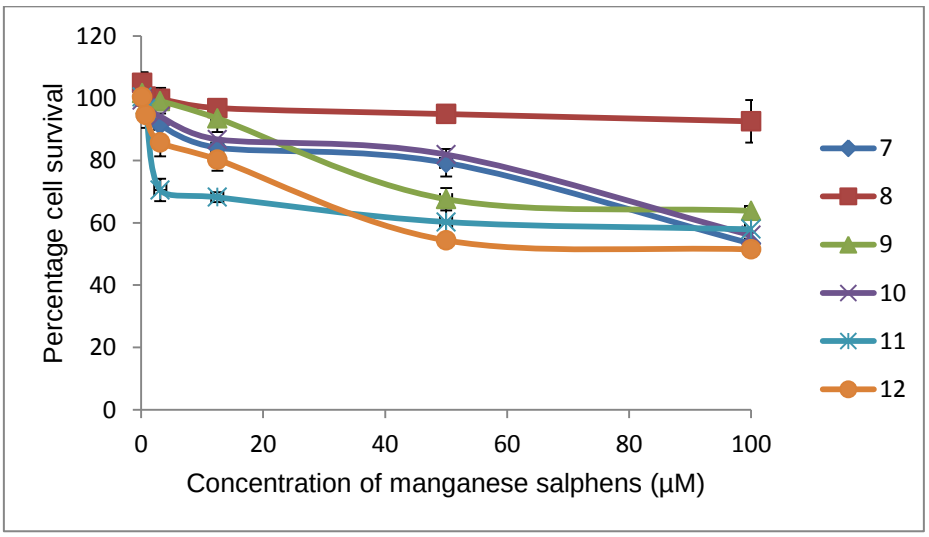


Figure 2-7 IC_{50} of Manganese (III) salphens (compounds 7-12) on HT22 cell line.

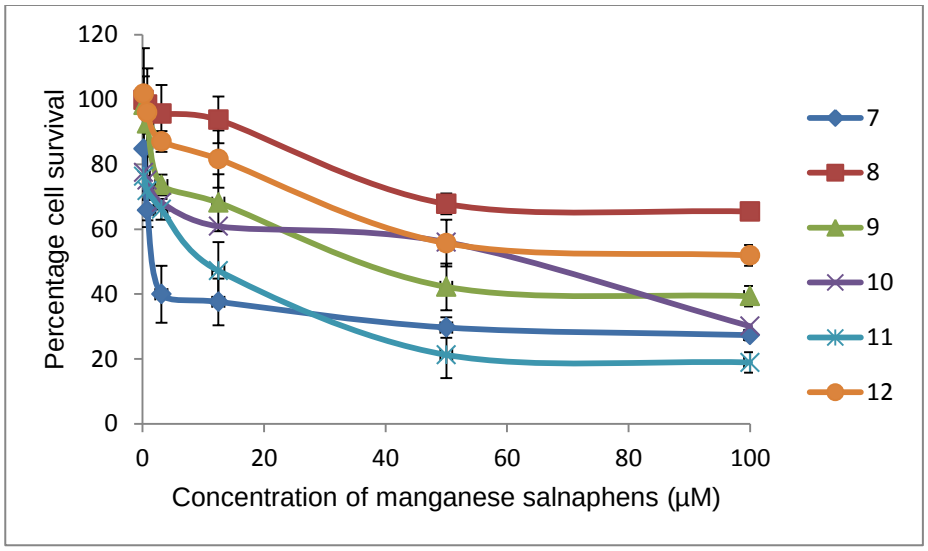


Figure 2-8 IC_{50} of Manganese (III) salnaphens (compounds 13-18) on SY-SY5Y cell line.

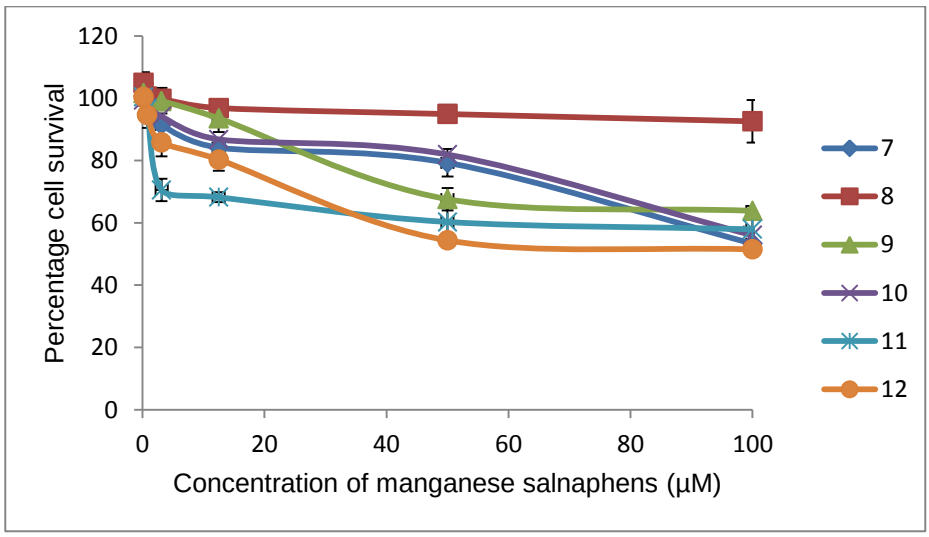


Figure 2-9 IC_{50} of Manganese (III) salnaphens (compounds 13-18) on HT22 cell line.

Viable cells percentage, relative to control (DMSO treated) cells were calculated using the absorbance (λ = 560 nm) values. The percent viable cells were plotted as a function of concentration and IC_{50} values were obtained. Each experiment was repeated thrice with four parallel replicates. Figures 2-4, 2-6, 2-8 show the percentage of viable cells relative to control for metallo-salens, metallo-salphens and metallo-salnaphens respectively on SH-SY5Y cell line. Figures 2-5, 2-7, 2-9 show the percentage of viable cells relative to control for the same compounds on HT22 cell line. The IC_{50} values for all the compounds from 1-18 are shown with standard error of mean (SEM) in Table 2-1. IC_{50} values of same compounds on MCF7 and MCF10 cell lines were taken from literature and compared with the obtained results. Most of the compounds showed a selective cytotoxicity towards cancer (SH-SY5Y) cells as compared to the HT22 cells. Fifteen out of eighteen compounds (compounds 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 14, 15, 16, 17 and 18) showed cytotoxic effects towards human neuroblastoma cells (SH-SY5Y) at very low concentrations with IC50 values ranging from 1.5 to 76 µM. Fifteen out of eighteen compound (1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17 and18) showed least effect on immortalized mouse hippocampal cell line (HT22).

The methoxy-substituted derivatives of Mn (III)-salens were found to be more effective. IC_{50} values of 3, 3' dimethoxy Mn (III)-salen and 4, 4' dimethoxy Mn (III)-salen was <5 µM. In Mn (III)-salphen series 3, 3' dihydroxy manganese (III)-salen and 4, 4' dimethoxy Mn (III)-salphen had IC_{50} values <10 µM. In Mn (III)-salnaphen series, 3, 3' dihydroxy Mn (III)-salen and 5, 5' dimethoxy Mn (III)-salphen had IC_{50} value <10 µM.

In general compounds 1, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 15 and 18 showed a potent toxicity towards SH-SY5Y cells and they were found to be better than cisplatin with IC_{50} 29 ± 3.5 µM. Upon changing the ethylenediamine bridge of parent manganese (III)-salen to more aromatic ortho-phenylenediamine and 2, 3-diaminonapthalene bridges, the cytotoxicity

effect of these compounds increased. These results indicate that aromatic ring plays an important role in determining the cytotoxic effects of Mn (III)-salen derivatives. In contrast compounds 15, 16, 17 and 18 were found to be active against both HT22 and SH-SY5Y cells, they lacked specificity.

2.6 Discussion

We analyzed the antitumor potential of several Mn (III)-salen derivatives and the results demonstrated that these metallo-salen derivatives affected cell viability and induced apoptosis in cultured human cancer cells. Studies showed that these compounds stimulated the release of cytochrome-c from mitochondria and translocation into the cytoplasm and activate caspase 3/7, leading to DNA fragmentation [51, 53, 57]. Chemotherapeutic agents including Mn (III)-salen derivatives have diverse mechanisms through which they exert their effects. For example, via intercalation of DNA, inhibition of DNA replication, cell membrane damage and free radical generation etc. The mechanisms, through which these compounds might act, still remain not completely understood. However, exposure of these compounds to the cells leads to the cell death of the SH-SY5Y cells via apoptosis. These results suggest that the Mn (III) salen derivatives are potential inhibitors of cell growth and proliferation in human cancer cells. This suggests that these compounds show some potential as anticancer drugs but *in vivo* characterization is required.

Chapter 3

ANTIOXIDANT PROPERTIES OF MANGANESE (III) SALENS UNDER OXIDATIVE STRESS

3.1 Introduction

Drugs with antioxidant properties are being widely proposed against several pathogenic disorders associated with oxidative damage caused by ROS (superoxide anion, hydroxyl radical and hydrogen peroxide). Metal compounds are well studied in drug research because they exhibit multiple oxidation states and can form stable complexes by coordinating with various ligands [2, 5]. Recent studies show that metal complexes with Schiff base ligands could potentially facilitate the scavenging of ROS. This can restore the redox balance in damaged cells and organs. Schiff's bases are straight forward to prepare and versatile. A well designed Schiff's base is considered as privileged ligands, they stabilize different metal ions in various oxidation states controlling the performance of metals in various clinical applications. Schiff's bases are largely studied because of their ability to bind reversibly with oxygen redox systems and oxidation of DNA. The C=N link is essential for bioactivity and its antioxidant properties [7]. These complexes closely mimic active site of antioxidant enzymes and the redox property of a metal ion in the complex is critical in their activity. The Manganese (II) complexes of pentaaza macrocyclic ligands are most thoroughly investigated molecules in an *in vivo* model. Most of these compounds showed antiarthritis, antitumor and pain relieving properties [7]. The molecular mechanism is not well understood, however different rate determining steps for superoxide dismutation have been proposed based on stopped flow or pulse radiolysis experiments [7]. Aromatic and substituted ethylenediamine complexes have been reported to have antioxidant properties. Manganese (III) complexes have been shown to scavenge $O_2^{\cdot-}$, H_2O_2 and lipid peroxides.

In these salens, manganese is coordinated to oxygen and nitrogen results in the formation of several possible valence states and these valence states play an important role in scavenging ROS. Based on the above features, several metallo-salen derivatives have been synthesized and screened for their toxicity in human cancer cells. Detailed biochemical analysis and in vivo studies are essential before considering any compound as potential. In this study we used ethanol to generate oxidative stress in the cells. Acetaldehyde generated in ethanol metabolism was shown to promote cancer by interfering with several mechanisms, with replication of DNA. It can cause point mutations to gross chromosomal alterations. It affects the DNA repair mechanism and can interact with DNA building blocks to form new molecules. These adducts may trigger replication errors and mutations in cancer causing genes. We treated cells with alcohol to generate oxidative stress and the treated then with the compounds to check their antioxidant potential.

3.2 Materials and Methods

All reagents for organic synthesis and buffers were purchased from Sigma-Aldrich unless otherwise noticed. Tissue culture medium DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), penicillin and streptomycin were purchased from Sigma–Aldrich. 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. SH-SY5Y and HT22 were obtained from ATCC (American Type Culture Collection). Fluostar-Omega, BMG, Labtech micro-plate reader was used to record the absorbance of 96 well titer plates following MTT assay.

3.3 MTT Assay and IC50 Determination of Ethanol

To evaluate the toxic effects of ethanol and optimize the concentration of ethanol for later use, MTT assay wwas performed using ethanol. Approximately 7,500 cells were seeded into 96 well micro titer plates (100 µL DMEM) and were grown to 50-70% confluence. The cells were then treated with 100 µL DMEM containing ethanol. The final concentrations in the wells were maintained between 0-1200 mM for 24-36 hours. Control cells were treated with 200 µL of DMEM and blank wells had only DMEM. The plate was then covered with parafilm to control evaporation of Ethanol. Each reaction was performed in 4 wells in parallel. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed after 24-36 hours of treatment. About 20 µL MTT (stock 5 mg/mL in phosphate buffer saline) was added into each well of micro titer plate and incubated for 2-3 hours. NAD(P)H-dependent cellular oxidoreductase enzymes in the viable cells convert MTT to formazan crystals. Media is then discarded and the formed formazan crystals were dissolved in DMSO and incubated for 2 more hours with continuous shaking. The absorbance is then measured at 560 nm using a micro plate reader (Fluostar-omega, BMG Labtech). Percentage viable cells relative to control were calculated using the absorbance values. The percent viable cells were plotted as a function of concentration to obtain the IC_{50} values. Each experiment was repeated twice to confirm the results.

3.4 Cytotoxicity And Antioxidant Properties Of Manganese (III) Salen Derivatives under Oxidative Stress

Approximately 5,000 cells were seeded into 96 well micro titer plates (80 µL DMEM) and were grown to 50-70% confluence. The cells were then treated with 20 µL of compounds 1-18 dissolved in DMSO. 100 µL of DMEM + ethanol were added into the

wells, the final concentration of ethanol was maintained at 350 mM and the drug concentration between 0 to 100 µM for 24 hours. Control cells were treated with same amount of DMSO and ethanol controls were treated with same amount of ethanol without any drug. 180 µL DMEM mixed with 20 µL of DMSO was used as blank. After adding ethanol the micro titer plate is sealed with parafilm to prevent ethanol from evaporating. Each reaction was performed in 4 wells in parallel. MTT assay was performed after 24 hours of treatment. 20 µL MTT (stock 5 mg/mL in PBS) was added into each well of micro titer plate and incubated for 2-3 hours. NAD(P)H-dependent cellular oxidoreductase enzymes in the viable cells convert MTT to formazan crystals. Media is then discarded and the formed formazan crystals were dissolved in DMSO and incubated for 2 more hours with continuous shaking. The absorbance is then measured at 560nm using a micro plate reader (Fluostar-omega, BMG Labtech). Percentage viable cells relative to control were calculated using the absorbance values. The percent viable cells were plotted as a function of concentration to obtain the IC_{50} values. Each experiment was repeated twice to confirm the results.

3.5 Cell Morphology, Nuclear Fragmentation Study With DIC And DAPI Staining

Changes in the cell morphology in the presence of Mn (III) salen and its derivatives were tested in presence and absence of ethanol. About 5000 cells were seeded in 60mm plates with a cover slip. After overnight growth the cells were treated with IC_{50} concentrations of MN (III) salen and its derivatives in presence and absence of ethanol. Cells were checked for varying time period (24, 48 and 72 hours). Control cells were treated with equivalent amount of DMSO. Cells were then fixed with 4% formaldehyde solution for 30 min, washed with PBS for 5min. Cells were permeabilized with 0.2% triton X-100 in PBS for 5 min. and washed with ice cold PBS, this process was

repeated 2 times. Cells were finally washed with 0.02% tween-20 solution and stained with DAPI (5 µL per slide) for 10 minutes. The cells were finally washed with PBS for 3 more times and mounted on a microscope slide with mounting media (Vectashield H-1000, Vector Lab) and visualized under fluorescent microscope.

3.6 Results

3.6.1 MTT Assay and IC50 Determination of Ethanol

Cytotoxicity experiments (IC_{50} values were obtained) were performed to optimize the ethanol concentration, suitable for analyzing the oxidative stress conditions. Ethanol concentrations between 0-1200 mM were analyzed in both SH-SY5Y and HT22 cells. Each experiment was performed three times in 4 parallel replicates. After adding ethanol to the wells at appropriate concentrations the plates were immediately sealed with parafilm to stop evaporation of ethanol. The cell viability was then tested using MTT assay after 24-36 hrs post treatment.

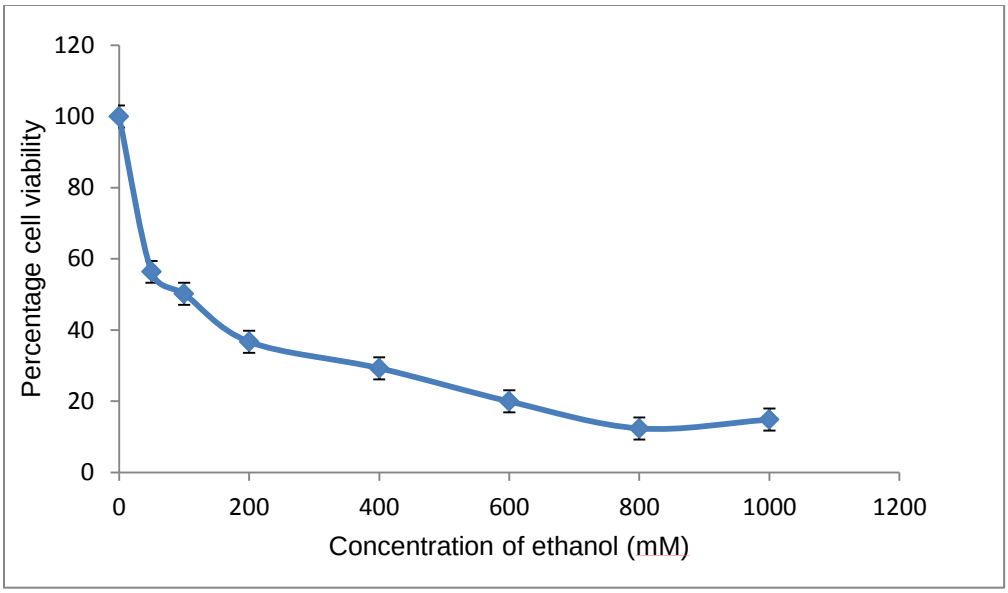


Figure 3-1 IC_{50} of Ethanol on SH- SYSY cell line.

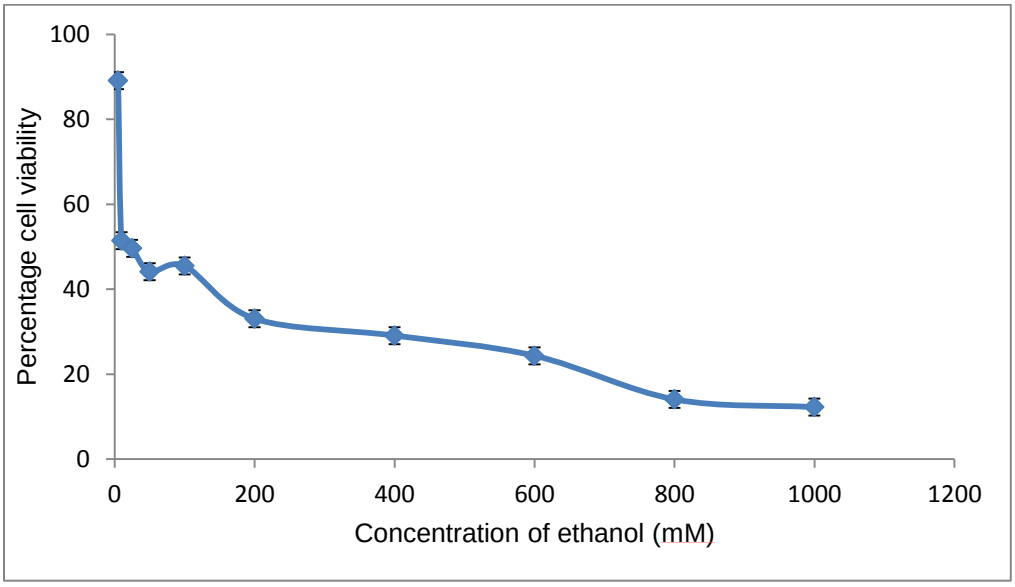


Figure 3-2 IC_{50} of Ethanol on HT22 cell line.

Percentage of viable cells relative to control were calculated using the absorbance values. The percent of viable cells were then plotted as a function of concentration of ethanol and IC_{50} values were obtained. Each experiment was repeated thrice to confirm the results. Figures 3-1 and 3-2 shows the scatter plot of percentage viable cells relative to non-ethanol controls on SH-SY5Y and HT22 cell lines, respectively. IC_{50} values were calculated and found that the IC_{50} for ethanol was 362 ± 1.7 mM in SH-SY5Y cell line and 335 ± 2.1 mM in HT22 cell line. These concentrations of ethanol were used in ethanol assays to evaluate the antioxidant properties of Mn (III)-salen derivatives.

3.6.2 Cytotoxicity And Antioxidant Properties Of Metallo-Salens Under Oxidative Stress

Tests for calculating IC_{50} were performed optimize the ethanol oxidative stress conditions. Approximately 5,000 cells were seeded into 96 well micro titer plates (80 µL DMEM) and were grown to 50-70% confluence. The cells were then treated with 20 µL of compounds 1-18 dissolved in DMSO. 100 µL of DMEM + ethanol were added into the wells, the final concentration of ethanol was maintained at 350 mM and the drug concentration between 0 to100 µM for 24 hours. Control cells were treated with same amount of DMSO and ethanol controls were treated with same amount of ethanol without any drug. 180 µL DMEM mixed with 20 µL of DMSO was used as blank. After adding ethanol the micro titer plate is sealed with parafilm to prevent ethanol from evaporating. The cell viability was then tested using MTT assay after 24-36 hours of treatment.

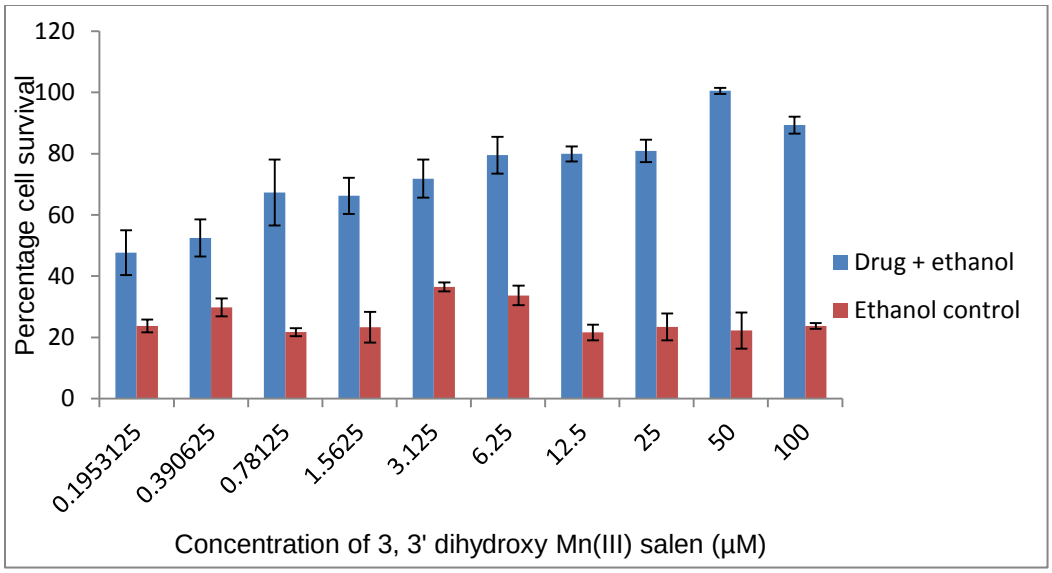


Figure 3-3 Ethanol assay of 3, 3’ dihydroxy Mn(III) salen on HT22 cell line.

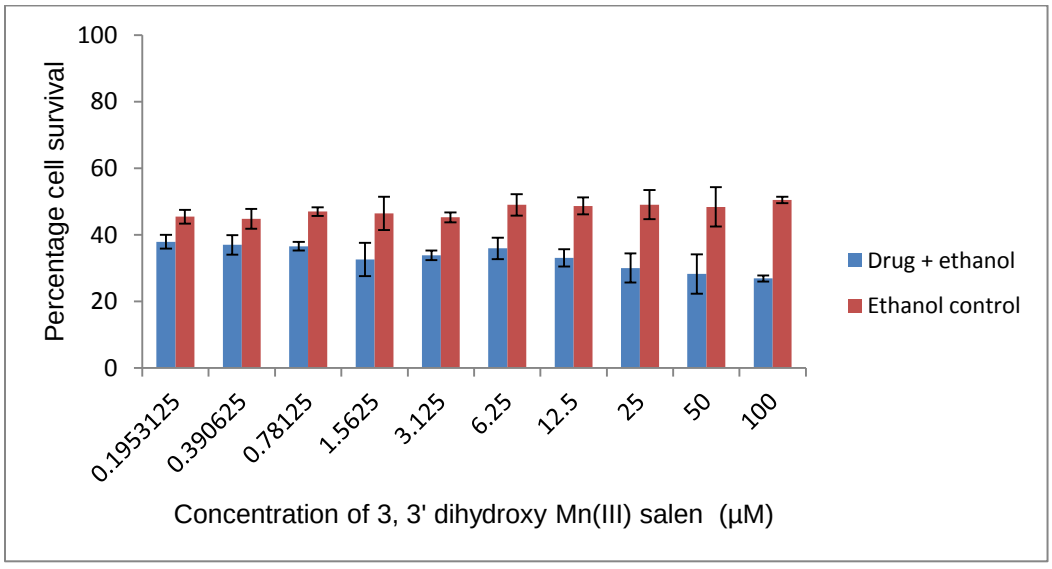


Figure 3-4 Ethanol assay of 3, 3’ dihydroxy Mn(III) salen on SH-SY5Y cell line.

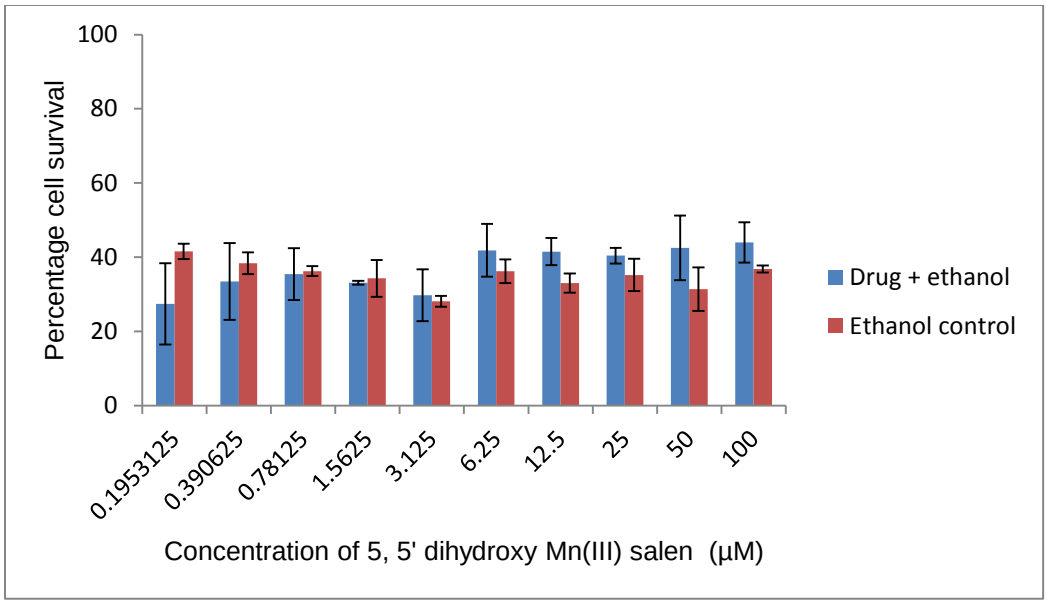


Figure 3-5 Ethanol assay of 5, 5’ dihydroxy Mn(III) salen on HT22 cell line.

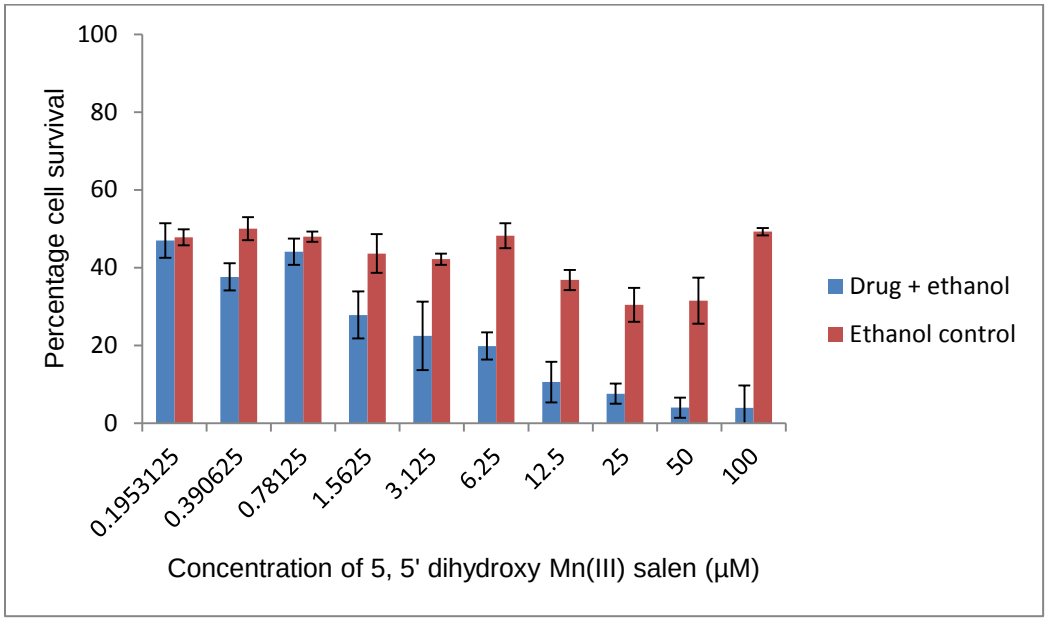


Figure 3-6 Ethanol assay of 5, 5’ dihydroxy Mn(III) salen on SHY cell line.

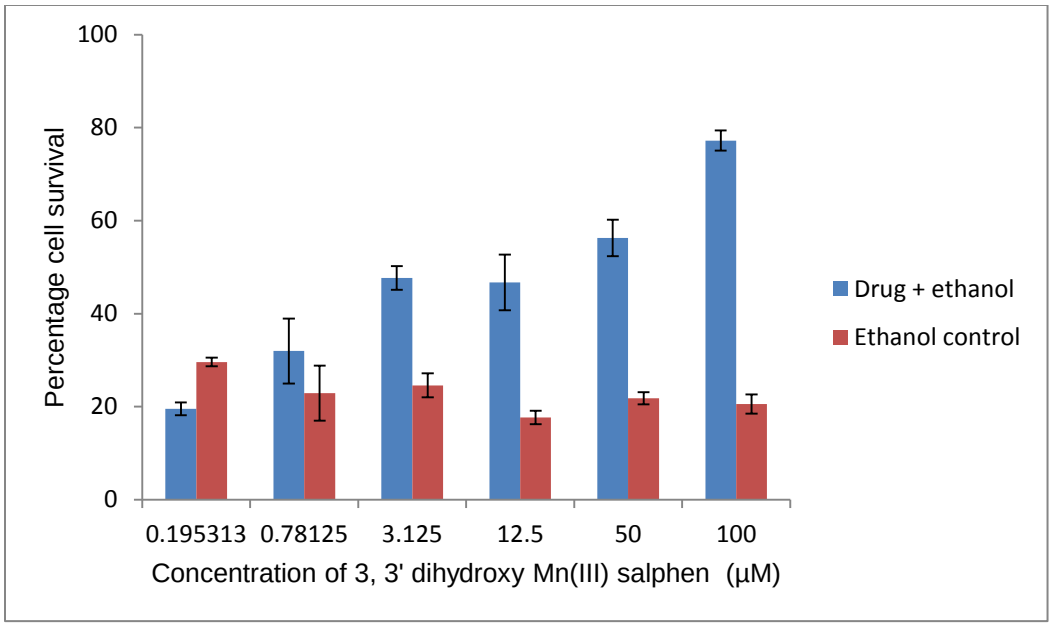


Figure 3-7 Ethanol assay of 3, 3' dihydroxy Mn (III) salphen on HT22 cell line.

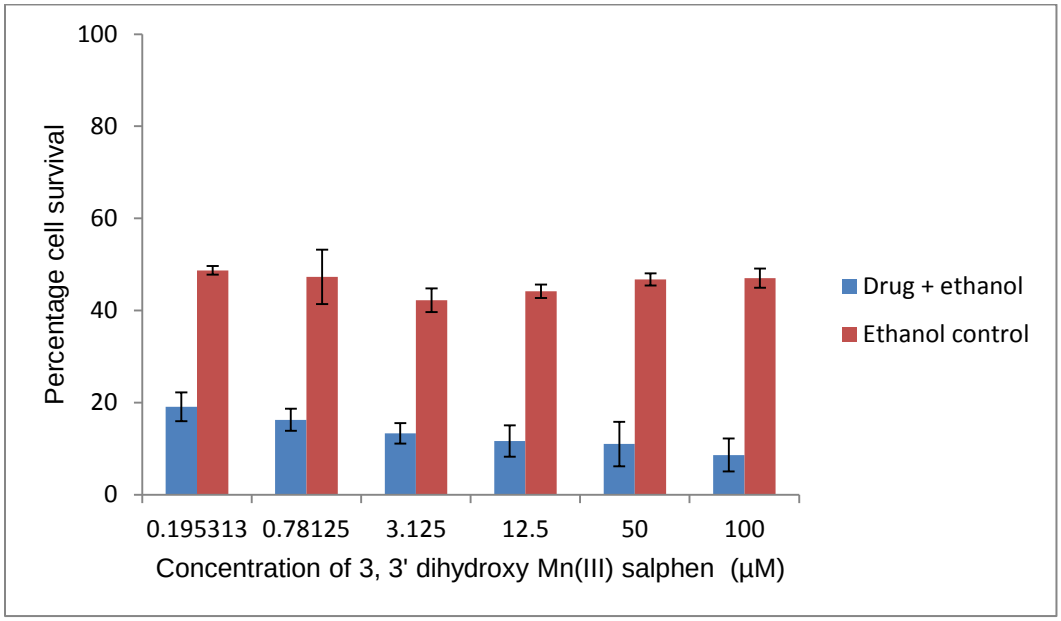


Figure 3-8 Ethanol assay of 3, 3' dihydroxy Mn (III) salphen on SH-SY5Y cell line.

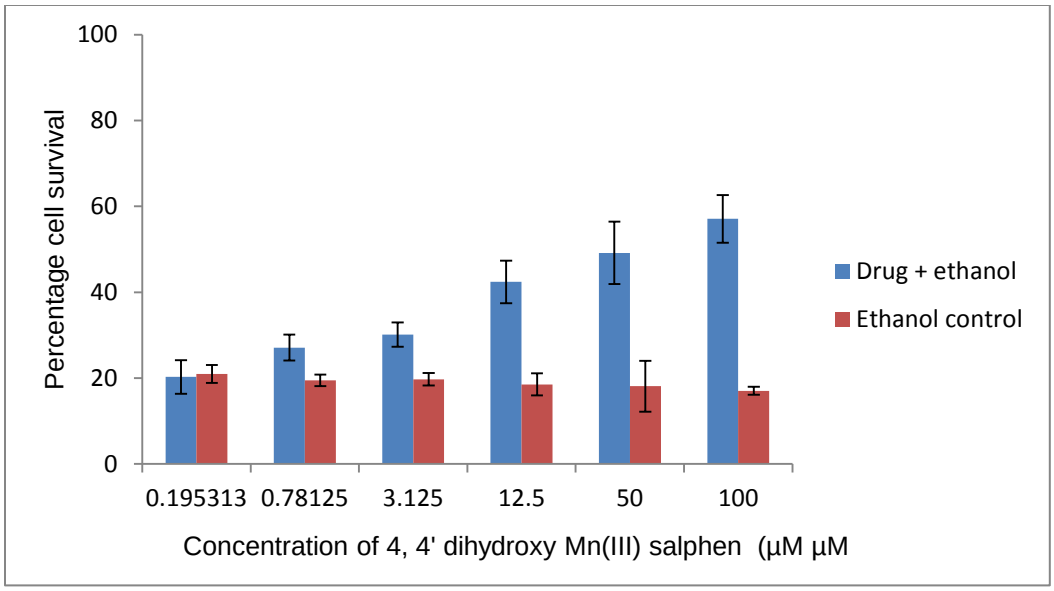


Figure 3-9 Ethanol assay of 4, 4' dihydroxy Mn (III) salphen on HT22 cell line.

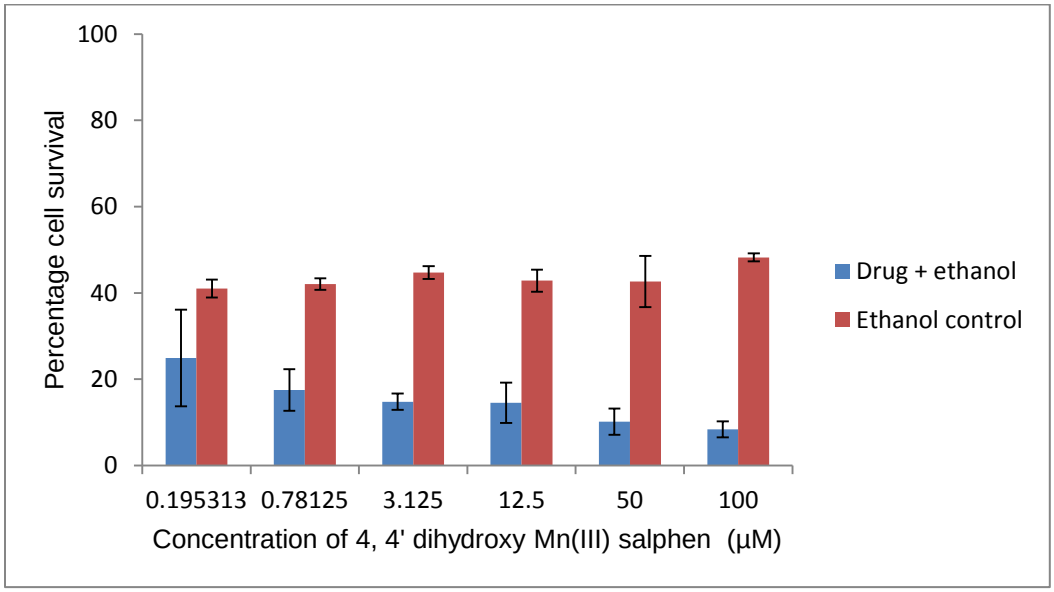


Figure 3-10 Ethanol assay of 4, 4' dihydroxy Mn (III) salphen on SH-SY5Y cell line.

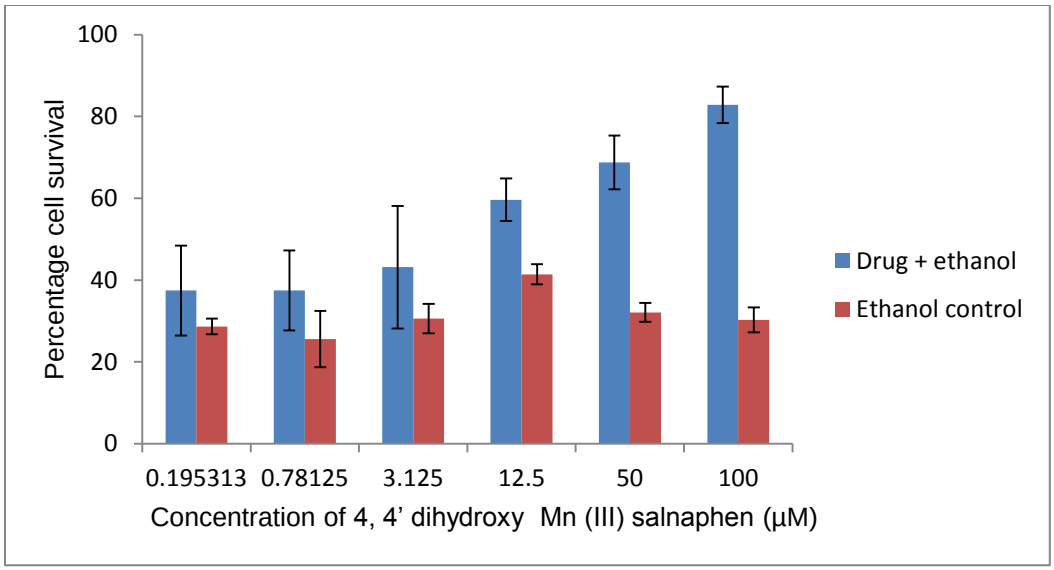


Figure 3-11 Ethanol assay of 4, 4' dihydroxy Mn (III) salnaphen on HT22 cell line.

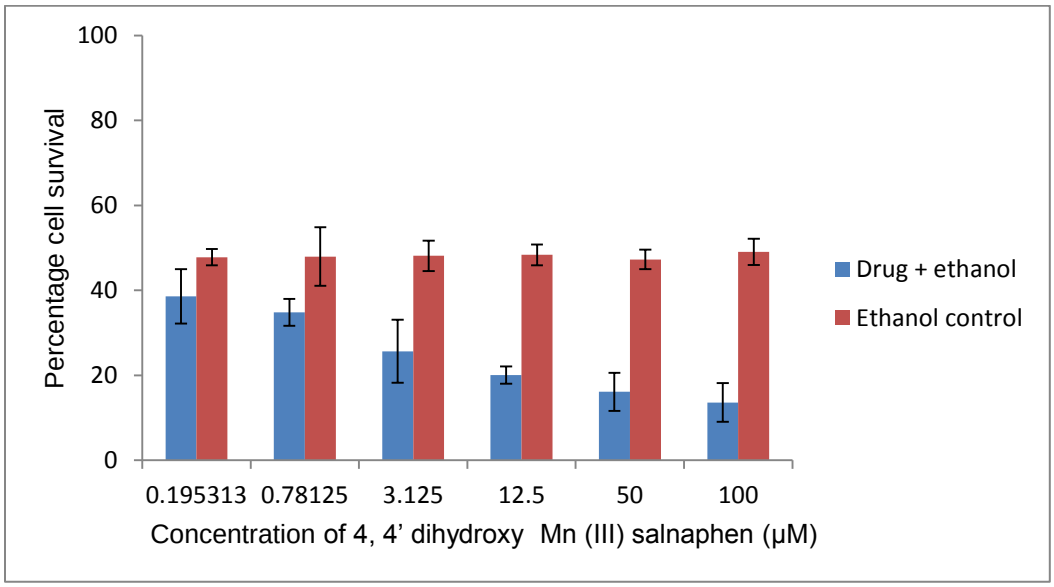


Figure 3-12 Ethanol assay of 4, 4' dihydroxy Mn (III) salnaphen on SH-SY5Y cell line.

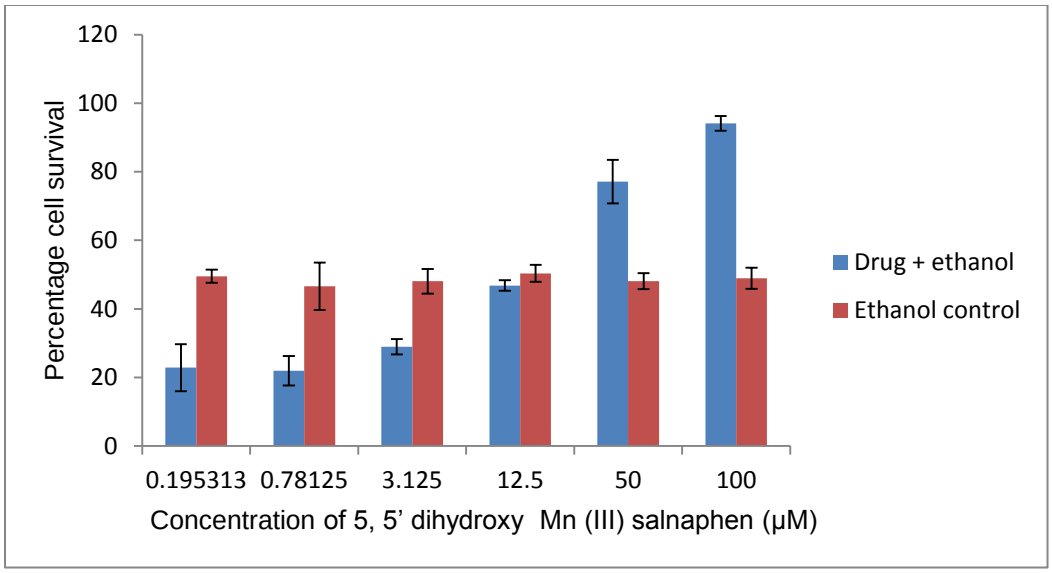


Figure 3-13 Ethanol assay of 5, 5' dihydroxy Mn (III) salnaphen on HT22 cell line.

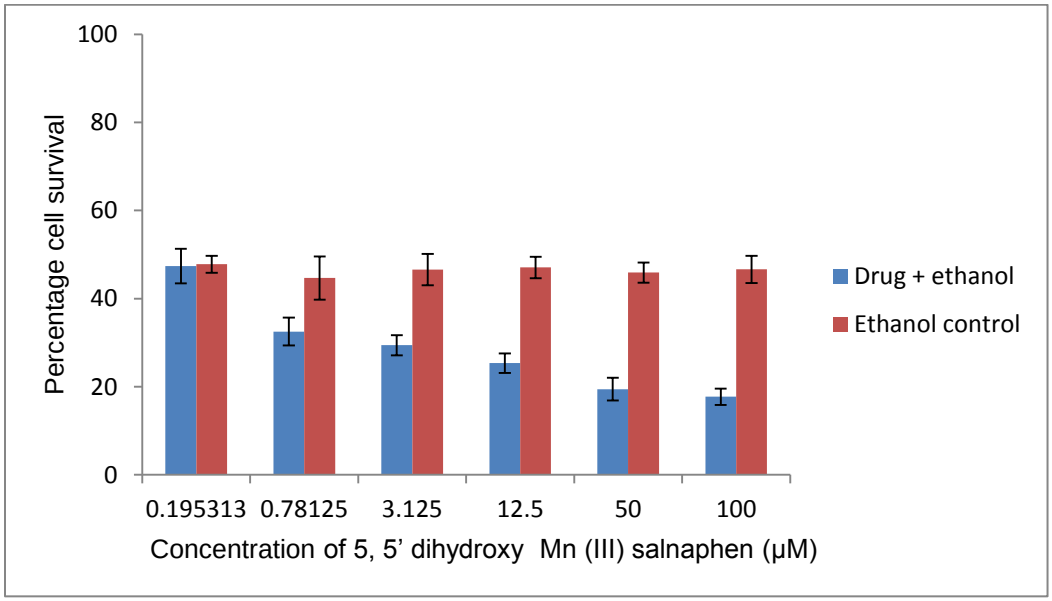


Figure 3-14 Ethanol assay of 5, 5' dihydroxy Mn (III) salnaphen on SH-SY5Y cell line.

The percent viable cells were then plotted as a function of concentration. Figure 3-3 shows the cell viability of HT22 cells in presence of ethanol and 3, 3' dihydroxy Mn (III)-salen. The cell viability of HT22 cells was found to increase with the higher concentration of the compound. The ethanol induced cell death was reduced in the presence of the compound. Cells viability was reduced by ~3 folds more as compared to cell viability in the ethanol control cells where they are exposed to ethanol alone. In contrast the same compound showed toxicity in SH-SY5Y cells and were not able to protect the cells from the ethanol induced oxidative stress. Figure 3-4 shows the cell viability in presence of 3, 3' dihydroxy Mn (III)-salen and ethanol. The cell viability decreased with increase in concentration of the compound. Control cells were treated with ethanol alone and cell viability in test was found to be one-third of the control cell viability. The same was trend was seen with almost all the compounds and data form 6 compounds (Compounds 1, 2, 7, 8, 13, 14 respectively) was shown above in figure 3-3 to 3-14. The dimethoxy derivatives didn't show much effect on both the cell lines. Most of the compounds showed a selective cytotoxicity towards cancer cells in presence of ethanol.

3.6.3 Cell Morphology, Nuclear Fragmentation Study With DIC And DAPI Staining

Nuclear fragmentation and alterations in the cell morphology in the presence of Mn (III) salen and its derivatives were tested in presence and absence of ethanol. Cells were treated with ethanol and drugs as mentioned in the procedure discussed above. Cells were then stained with DAPI and visualized under fluorescent microscope using 100X oil immersion and 40X objectives. Differential interference contrast (DIC) images were taken to study the cellular morphology (Figures 3-15 to 3-18).

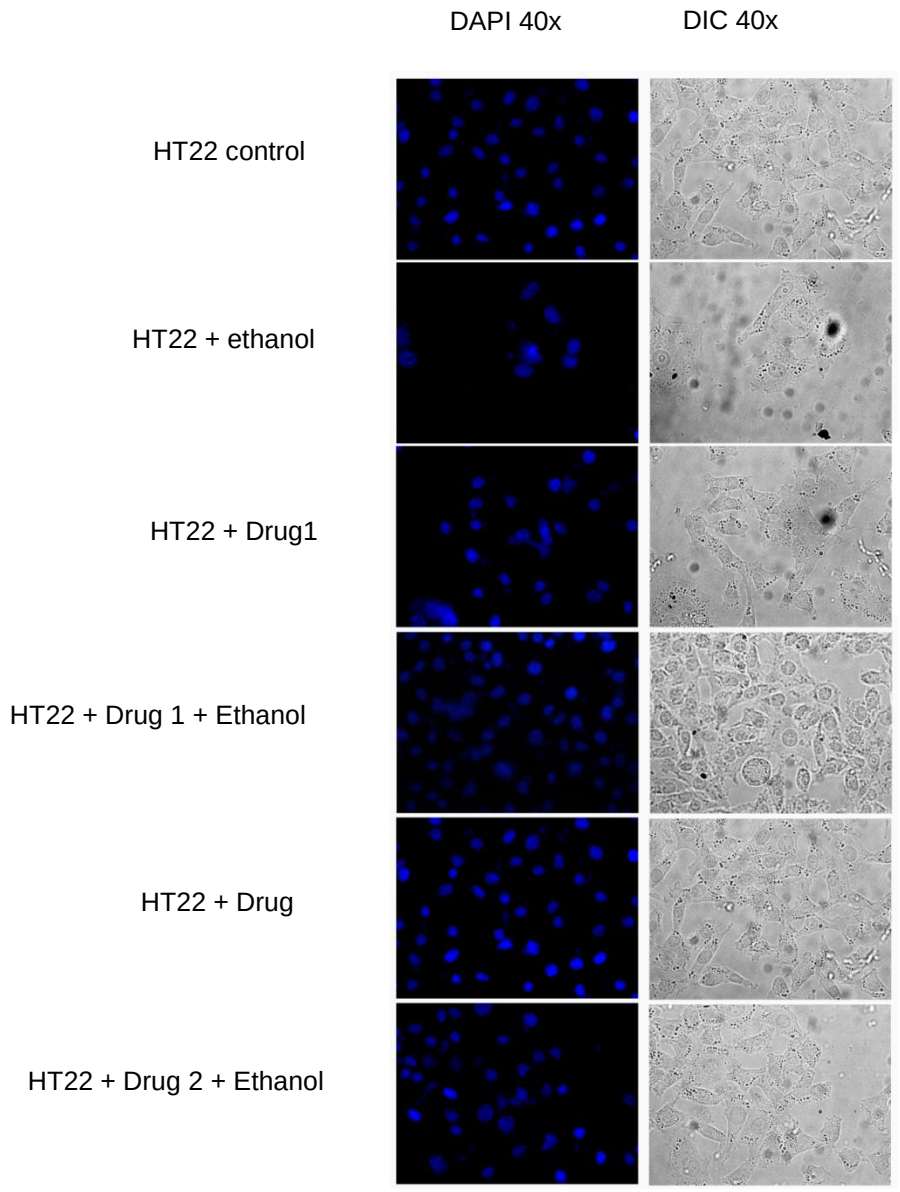


Figure 3-15 DAPI and DIC images of HT22 cells before and after treatment with drug 1, 2 and ethanol.

Drug 1: of 3, 3’ dihydroxy Mn (III) salen

Drug 2: of 3, 3’ dihydroxy Mn (III) salphen

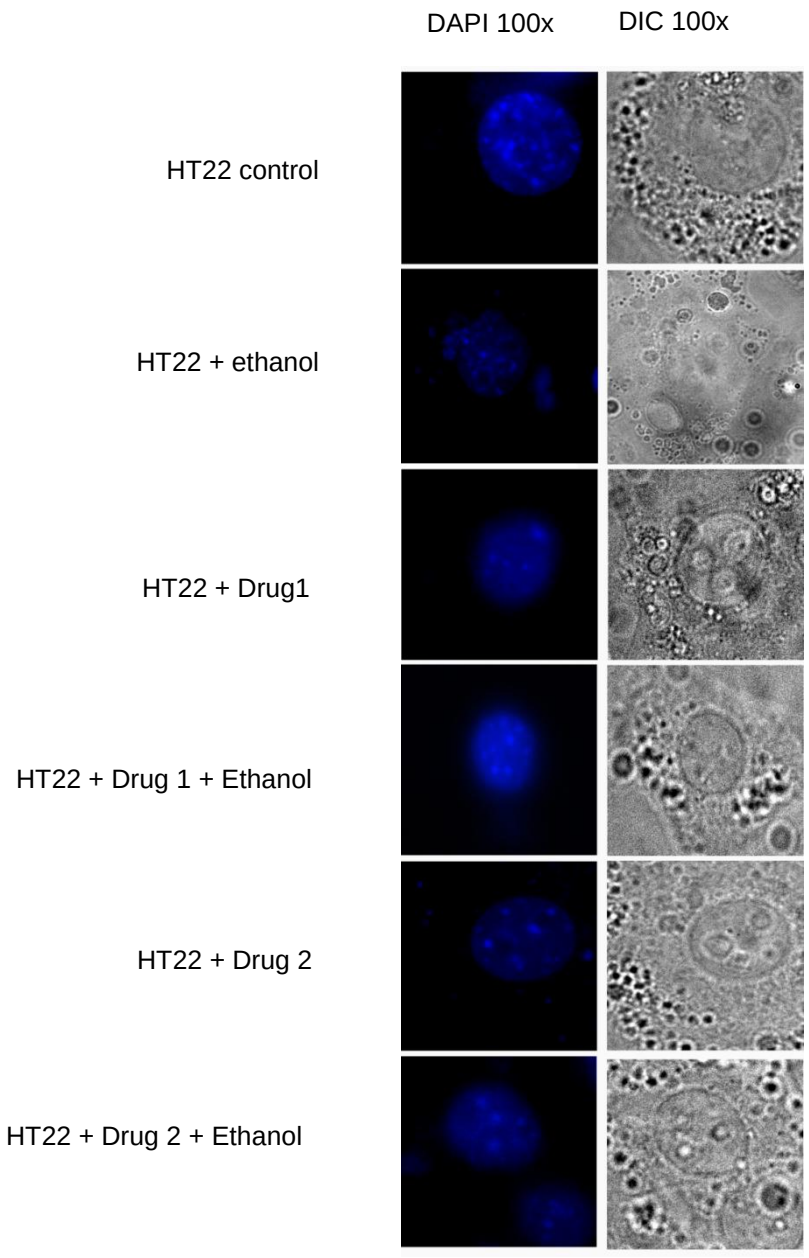


Figure 3-16 DAPI and DIC images of HT22 cells before and after treatment with drug 1, 2 and ethanol.

Drug 1: of 3, 3' dihydroxy Mn (III) salen

Drug 2: of 3, 3' dihydroxy Mn (III) salphen

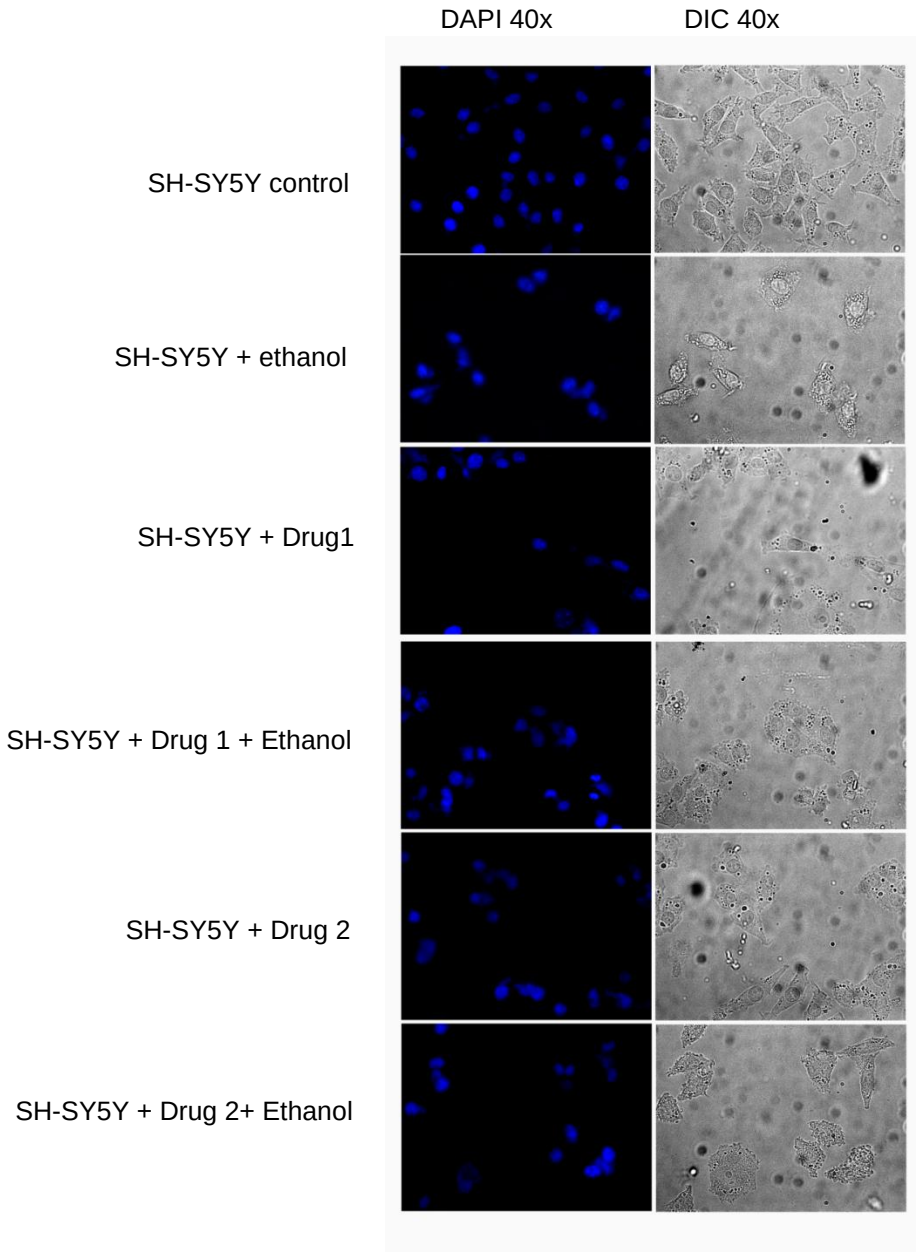


Figure 3-17 DAPI and DIC images of SH-SY5Y cells before and after treatment with drug 1, 2 and ethanol.

Drug 1: of 3, 3’ dihydroxy Mn (III) salen

Drug 2: of 3, 3’ dihydroxy Mn (III) salphen

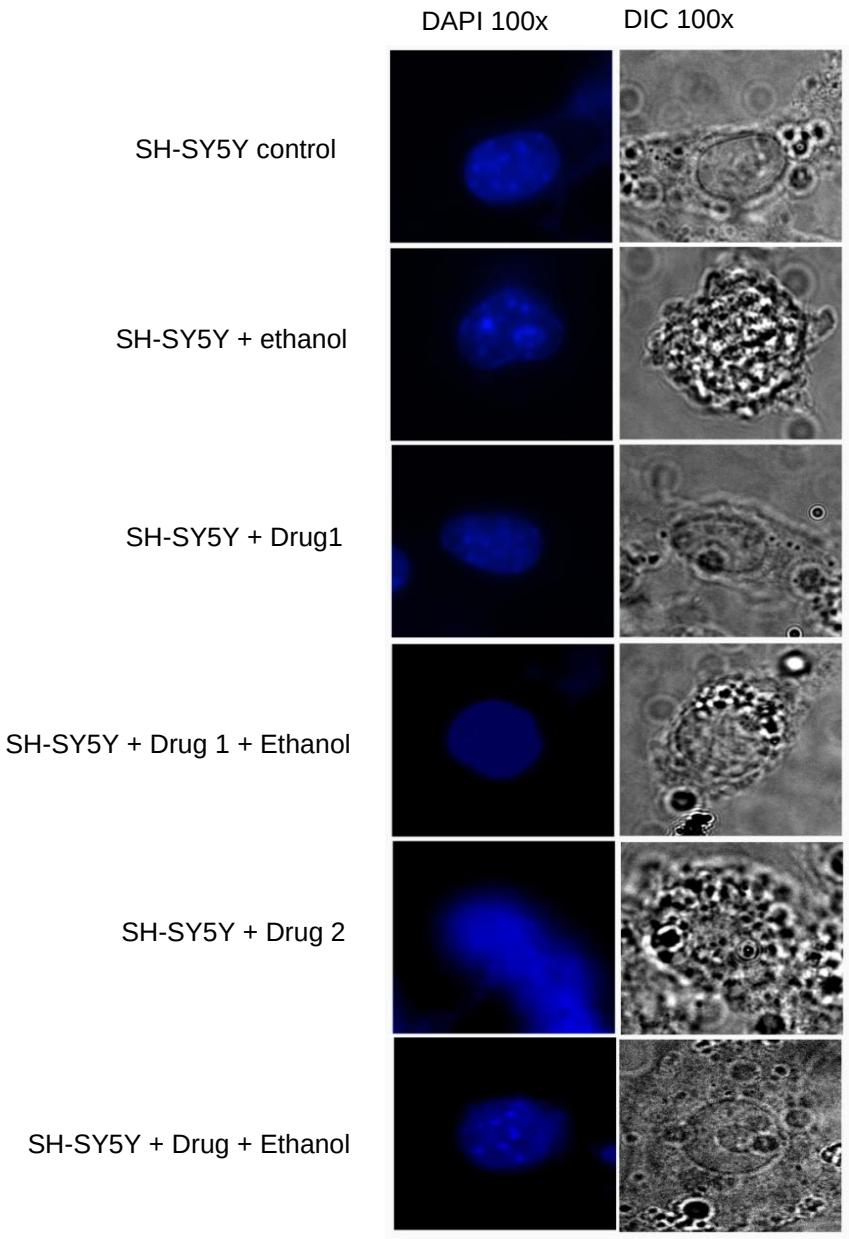


Figure 3-18 DAPI and DIC images of SH-SY5Y cells before and after treatment with drug 1, 2 and ethanol.

Drug 1: of 3, 3’ dihydroxy Mn (III) salen

Drug 2: of 3, 3’ dihydroxy Mn (III) salphen

Cells exposed to 2 compounds (3, 3' dihydroxy Mn(III)-salen, 3, 3' dihydroxy Mn(III)-salphen) displayed apoptotic alterations such as shrivelling of the cell surface, internucleosomal DNA fragmentation and even deformed nuclear structure. Figure 3-15 shows the effect of these compounds in presence and absence of ethanol at 40X magnification on HT22 cell line. The cell viability was not affected and cells tend to grow normally even in the presence of ethanol as compared to the control cells. Figure 3-16 shows the effect of these compounds in presence and absence of ethanol at 100X magnification. The nucleus of the cells was found intact. The DIC images showed the normal cell morphology in presence of ethanol and compounds. Cell disruption was not seen in the treated cells where as in presence of ethanol alone cell viability was less and the cell morphology was slightly round indicating that cells are not healthy. Even nuclear fragmentation was seen in presence of ethanol alone. Figure 3-17 shows the effect of these compounds in presence and absence of ethanol at 40X magnification on SH-SY5Y cell line. The cell viability was found to be very less in the ethanol treatment alone and ethanol with the compounds. The cells morphology was disrupted and cell debri was seen. Figure 3-18 shows the 100X magnification of these cells in presence of ethanol treatment. The cells looked more circular and nuclear fragmentation is seen in presence of compounds and ethanol. These show that the compounds were specific towards the cancer cells.

3.7 Discussion

These compounds not only protected HT22 cells from ethanol mediated oxidative stress but also were selectively toxic to the cancer cells at the same time. Most of the compounds showed a selective cytotoxicity towards cancer cells. The dihydroxy derivatives were toxic to the cancer cells in presence of ethanol and they didn't kill any of the normal cells rather the cell growth was enhanced. These results suggest that these

compounds are potential antioxidants with anti-cancer properties. The molecular mechanism through which selective toxicity is achieved requires further studies, since the molecular mechanisms of these novel compounds still remain elusive. However different rate determining steps for superoxide dismutation have been proposed based on stopped flow or pulse radiolysis experiments [7]. These drugs need further *in vivo* characterization.

3.8 Summary and Conclusion

Large number of antineoplastics are being synthesized and screened every year. Only few of them go into clinical trials and after a decade or so, 2-3 compounds come into market. Drugs currently available have several adverse effects and are limited to certain types of cancer. Most of the active research is concentrated on producing anticancer drugs with less severe side effects. Design of such drug is a challenge as it is difficult to predict the physiochemical interactions of these complexes in body.

Biochemical evaluation of a drug is needed to find the mode of action and pathways involved so that they can be used to selectively target cancer cells. In this context, a total of 18 different compounds were synthesized and evaluated for their cytotoxicity. These compounds induce apoptosis in SH-SY5Y cells by condensation and fragmentation of nucleus which is observed from DAPI staining. Seventeen out of twenty-two compounds showed cytotoxic effects towards human neuroblastoma cells (SH-SY5Y) at very low concentrations with IC50 values ranging from 1.5 to 76 µM. Eighteen out of twenty two compounds didn't show any effect on immortalized mouse hippocampal cell line (HT22). These compounds were also evaluated for their antioxidant properties. Ethanol assay showed that 10 of 18 compounds showed potent effect on cancer cell lines and didn't show any effect on normal cells (HT22). The cell morphology was observed

with DIC microscopy and it revealed nuclear condensation in neuroblastoma cells. The cell growth was minimal in presence of ethanol, but when HT22 cells were treated with drug and ethanol, their growth was normal. These compounds can be novel drug candidates for targeting cancer cells specifically and to reduce oxidative stress in healthy cells.

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