ANTIOXIDATIVE AND ANTINEOPLASTIC EFFECTS OF Mn(III) SALPHENS, S-ALLYL CYSTEINE AND ITS SYNTHETIC ANALOGUES

by

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Abstract

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Presently used chemotherapeutic drugs are not very selective and do not have the capability to differentiate between normal cells and malignant cells, which leads to the development of adverse side-effects and systemic toxicity. The primary goal of this study is to discover a novel drug which can be used in the treatment of cancer as a chemotherapeutic drug. Here we have synthesized and screened 14 different compounds (12 metallo-salen complexes and 2 amino acid based compounds) and evaluated their cytotoxicity on human neuroblastoma cell line (SH-SY5Y) and mouse hippocampal cell line (HT22). Out of the 12 metal-complexes, 5 complexes had a better cytotoxic effect on the neuroblastoma cell line with IC_{50} value in the range 1.5 - 64 μM in SH-SY5Y cell line. 9 out of the 12 metallo-complexes showed low cytotoxicity towards the hippocampal cell line HT22. The same set of metallo-salen complexes and amino acid derivatives (S-allyl cysteine (SAC) and its derivative S-benzyl cysteine (SBC)) were screened for their

antioxidant activities in HT22 cells using an ethanol-induced oxidative stress based antioxidant assay. These analysis demonstrated that several metallo-salens, SAC and SBC showed protective effects in HT22 cells in the presence of ethanol. The compounds 3,3'-dihydroxy Mn(III) salphen (**5**) and 4,4'-dimethoxy Mn(III) salphen (**9**) also showed inhibition of colony formation in the case of SH-SY5Y cell line, with the compound 9 being the most effective. Overall, our studies demonstrate novel cytotoxic and neuroprotective effects of metallo-salen derivatives, SAC and SBC.

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List of Abbreviations

DNA - Deoxyribose Nucleic Acid

RNA - RiboNucleic Acid

ROS - Reactive Oxygen Species

VEGF - Vascular Epithelial Growth Factor

NAMI-A - New Anti-tumor Metastasis Inhibitor-A

ATP - Adenosine TriPhosphate

GTP - Guanosine TriPhosphate

cAMP - cyclic Adenosine MonoPhosphate

cGMP - cyclic Guanosine MonoPhosphate

APAF-1 - Apoptotic Protease Activating Factor-1

TNF - Tumor Necrosis Factor

DISC - Death Initiating Signaling Complex

FADD - Fas-Associated Death Domain

AGE - Aged Garlic Extract

SAC - S-Allyl Cysteine

SBC - S-Benzyl Cysteine

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

Chapter 1

INTRODUCTION

1.1. History of metal complexes in medicine

Metal ions are known to play very critical roles in the biology of living systems especially in the stabilization of biomolecules like proteins and nucleic acids. The presence or absence of certain metal ions in the active site of the enzymes play a very important role in the activity of the enzyme, which in turn plays an important role in the physiological response of a particular organ system by serving as electron donors/acceptors or structural regulators of the active site of the enzyme.[1] Carboxypeptidase A, a pancreatic exopeptidase which is involved in the hydrolysis of peptide bonds of C-terminal region of proteins with aromatic or aliphatic side-chains and employs a zinc ion located within the protein to hydrolyze the peptide bond at the C-terminal end of an amino acid residue. Loss of the zinc ion from the active site of this enzyme results in the loss of activity of the enzyme.[2] Another example of metal ion mediated enzyme activity is liver alcohol dehydrogenase. This enzyme consists of two zinc ions: one is involved in the activity of the enzyme by coordinating with the substrate (alcohol); the other zinc ion is involved in maintenance of the structural stability of the enzyme.[3] There are a plethora of interactions between metal ions and DNA ranging from basic binding of the metal complexes to the DNA to neutralizing the negative charge on the DNA and hence unfolding the DNA from its tightly wound structure to make it accessible for transcription and gene regulation.[4] Therefore, deficiency of metal ions or

the imbalances in the level of these metal ions might result in diseases of varying severity from mild anaemia to infertility to even death.[5]

The use of metals in medicine dates back to as far as 3000 BC, where ancient Egyptians used copper sulphate to sterilize the water used in the tonics. Gold was used in dentistry, zinc was used to promote healing of wounds, Mercury (I) chloride was used as a tonic called "blue mass."[5] In the early 20th century, metal complexes were used in medicine and the screening process became more advanced which resulted in the production of arsphenamine (salvarsan), which was the first modern chemotherapeutic agent to treat syphilis. This marked the beginning of the discovery of a wide range of chemotherapeutic drugs, especially for their anticancer and antioxidant properties.[6] The congregation of inorganic chemistry with biochemistry resulted in the production of thousands of potential drugs with anticancer and antioxidant properties. Many of them are in clinical use currently even though they have damaging side effects and hence, there is an enormous amount of research being pumped into the discovery of drugs that have the least damaging effects on the nearby healthy tissue.[7]

1.2 Metal complexes as anticancer agents

Cisplatin, discovered in the late 19th century is the first anti-cancer drug and was called Peyrone's salt.[8] This was discovered accidentally while studying the effects of electric field on the growth of bacteria. But cisplatin was first reported by Rosenberg in 1965, later approved by the FDA in 1978 and was first used to treat testicular cancer.[9; 10] Other platinum based drugs that gained popularity were carboplatin, oxaliplatin,

satraplatin, picoplatin, nedaplatin, triplatin and lipoplatin.[11] The chemical structures of these compounds are shown in Fig 1-1.

Cisplatin is absorbed into the cells by passive diffusion and is hydrolyzed into an active diaqua complex as described in Scheme 1-1.[12] This complex binds to adjacent guanine bases and crosslinks with DNA making it non-replicable, thereby forcing the cell to undergo apoptosis. But the drug is associated with side effects like gastrointestinal and hematological toxicity due to its non-specificity and this problem is not just in case of cisplatin, but is also inherent in the other presently used chemotherapeutic drugs.[13] So, there is a need for discovering new synthetic chemotherapeutic drugs that are not associated with side effects.

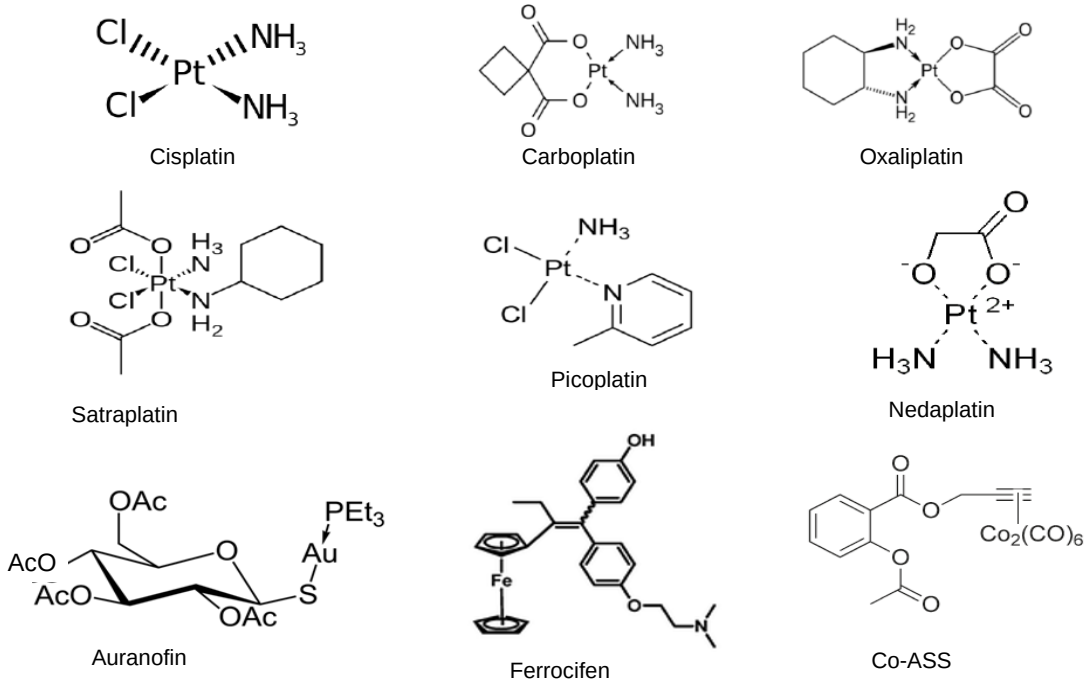
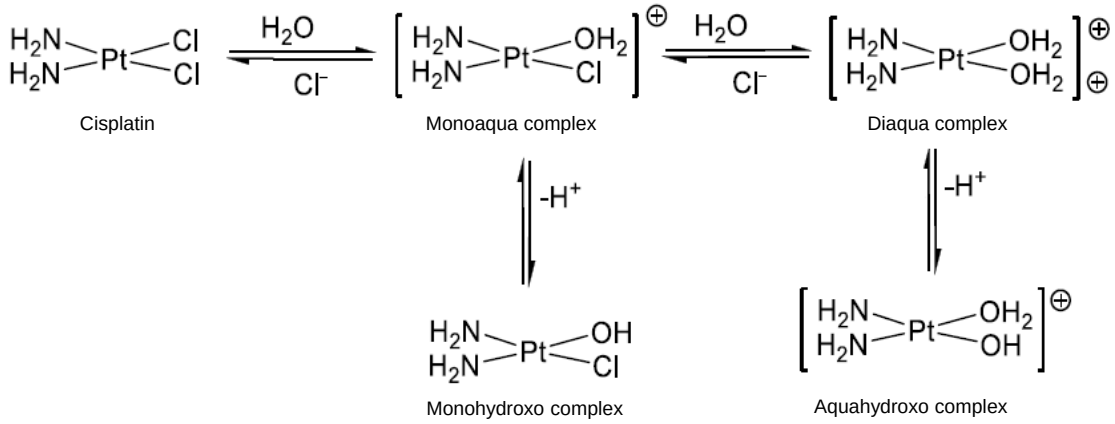


Fig 1-1 **Some of the metal complexes used in medicine**.[14] Metal complexes have been used in medicine for a long time and platinum, gold, iron, ruthenium and cobalt complexes have been used in anti-neoplastic chemotherapeutic drugs.



Scheme 1-1 **Activation of cisplatin in the cell.** Once cisplatin enters the cell, it is hydrolysed to monoaqua complex which is again hydrolysed to diaqua complex, which is the active form of cisplatin. This undergoes a dehydration when covalently bound with the nitrogen at the 7th position of adenosine or guanosine and activates apoptotic machinery due to DNA damage [Redrawn from [15]].

Most of these metal complexes, due to their ligand binding affinities and a spectrum of oxidation states have the ability to generate reactive oxygen species, that are involved in targetting the DNA and causing apoptosis.[16; 17] Similar mechanisms are followed by many other transition metal complex based chemotherapeutic drugs. For example, substituted ferrocenes undergo oxidation to form ferrocenium ion (Fe^{3+}) in the cells and damages the DNA by generating ROS.[17] A rather unusual route instead of ROS-mediated DNA damage is followed by cobalt based drugs. It has been shown that Co(III) complexes have the ability to cleave DNA and proteins under photolytic conditions.[18] This is because upon excitation by light, cobalt is excited from Co(II) to Co(III), which is basically involved in the cleavage of macromolecules such as proteins and DNA.[19] Gold based drugs are a burgeoning class of metal complexes with cytotoxic properties and are speculated to be potential antitumor agents. One of the major stimulant for

research into gold complexes is their stability under physiological conditions.[20] They are also a viable and a less expensive alternative to platinum based drugs since both Au(III) and Pt(II) have the same outer shell electronic configuration, d^8, preferentially favoring square planar complexes.[21] A widely used gold complex called auranofin was the first gold complex that was identified to have anti-cancer activity on HeLa cells.[21] The anti-cancer is due to the exchange of ligand with selenocysteine and cysteine residues in the active site of the antioxidant protein thioredoxin, resulting in the inhibition of the enzyme activity.[21] Even though gold is comparatively cheaper than platinum, the cost was still a barrier to both research and mainstream medicinal use and hence the interest shifted to other transition metal complexes as potential chemotherapeutic drugs.

Therefore, focus shifted to titanium, ruthenium and gallium complexes as potential anti-proliferative drugs. For example 9-budotitane and the metallocene, titanocene dichloride have been in clinical trial for their cytotoxicity in cancerous tissues.[22; 23] Ruthenium complexes have been studied extensively since the 1970s for their anti-cancer properties and two of the most common and successful Ruthenium-based drugs include NAMI-A and KP1019, the latter having a better inhibition of growth on the cultured cancer cells compared to the former.[24; 25] But the opposite was true in *in vivo* studies and NAMI-A was shown to suppress the expression of Vascular Endothelial Growth Factor (VEGF), which is mainly involved in angiogenesis.[26] Gallium based complexes were observed to play a role in the structure and biosynthesis of DNA and proteins. It was also observed to inhibit enzymes like DNA polymerase, several ATPases and disturb mitochondrial functions.[27] Gallium based drugs are usually given as a

combination therapy, where it is administered with vinblastine and ifosamide to treat patients with ovarian cancer.[28]

Eventhough the compounds are shown to have a considerable amount of specificity *in vitro*, the *in vivo* results were not as convincing. The ROS produced by these complexes upon interaction with cellular contents are capable of damaging nearby normal tissues. Hence there is a need for developing drugs that are antineoplastic with minimal side effects.

1.3 Metallo-salens, -salphens and -salnaphens as potential anti-cancer drugs

Research into metallo-salens induced a huge interest to exploit them as potential anticancer drugs and/or antioxidant drugs. They were classified as nucleases that had the capability to induce DNA-cleavage and apoptosis in cultured human cells [29; 30] and were shown to intercalate with the DNA and induce DNA cleavage, thereby damaging the DNA. They were also shown to target the cells with high specificity due to their flat electronic structure.[31; 32] The metallo-salens bring about DNA cleavage via different mechanisms. For example, Mn(III) salens and Ni(II) salens cleave the DNA using the help of terminal oxidants, but Co(II) salen and Cu(II) salen depend on a reducing co-factor to initiate DNA cleavage.[33] This promoted interest in our lab (Dr. Mandal lab) to synthesize and evaluate the activities of different -salen, -salphen and -salnaphen complexes. We have previously shown that Mn(III)-salen and -salphen complexes have antiproliferative activities and apoptotic activities in MCF7 cells.[34] Metallo-salens induce apoptosis through the mitochondrial pathway.[34] Some of the metallo-salen do exhibit

tumor selective apoptosis over normal cells.[34] Although the complexes are active in *in vitro* studies, a thorough and more detailed studies are needed to explore their potential application as chemotherpauetic drugs.

1.4 Cell death machinery and potential targets of anticancer agents

Programmed cell death or Apoptosis is a physiological process that leads to the death of cells and subsequent clearance of the cells by phagocytes. There are three pathways cells take to induce cell death viz. mitochondrial or intrinsic pathway, death receptor dependent or extrinsic pathway and perforin or granzyme mediated pathway. So, the proteins in these pathways are potential targets for candidate drugs.

The ideal anti-cancer agents that are involved in damaging the mitochondria induce mitochondrial permeability transition pore formation, thereby increasing the permeability of the mitochondrial membrane. The inner membrane proton gradient starts to dissipate and the outer membrane enhances the leakage of proteins that are usually localized in the intermembrane space. The pore formation is usually assisted by pro-apoptotic proteins like Bax and finally results in the rupture of the outer membrane of the mitochondria.[35] This releases the holocytochrome-c which induces the oligomerization of APAF1 (apoptotic protease activating factor 1), which then binds to the cytosolic procaspase-9 and assembles the apoptosome complex by juxtaposing two procaspase-9 molecules, resulting in the release of mature caspase 9 as shown in Fig 1-2. Capsase-9 then activates its downstream targets such as caspase-3 and caspase-7 which initiate the reactions leading to cell damage.[35; 36]

The death receptor Fas, a 45 kDa protein is a member of the Tumor Necrosis Factor (TNF) receptor superfamily. The ligand that binds to the Fas receptor (FasL) induces the clustering of Fas receptor with adaptor proteins and forms a complex called death-initiating signaling complex (DISC) which involves the adaptor protein Fas-associated death domain (FADD) and procaspase-8.[35] FADD binds to the cytoplasmic region of the receptor and through the death domain and to the N-terminal domain of the procaspase-8 via the death effector domain (DED). Caspase-8 is activated when procaspase 8 oligomerizes with DISC, which may activate the downstream effector caspases or cleave the proapoptotic member of the Bcl-2 family.[35]

Usually T-cell mediated cell death is through the extrinsic pathway, but in tumor cells or virus infected cells, they follow a unique pathway which involves the secretion of perforin thereby causing the formation of the pore on the membrane followed by the release of granules through the pore into the target cell.[37] The granules that are released contain granzyme A, which has serine protease activity and granzyme B, which cleaves the proteins at aspartate residues activate the procaspase-10 and can cleave the inhibitor of caspase activated DNase (ICAD).[38] Granzyme B also directly activates the execution caspase (caspase-3) bypassing the upstream signaling pathways. Granzyme A helps in T-cell induced apoptosis in a caspase-independent fashion by nicking the DNA with the help of DNase NM23-H1.[39]

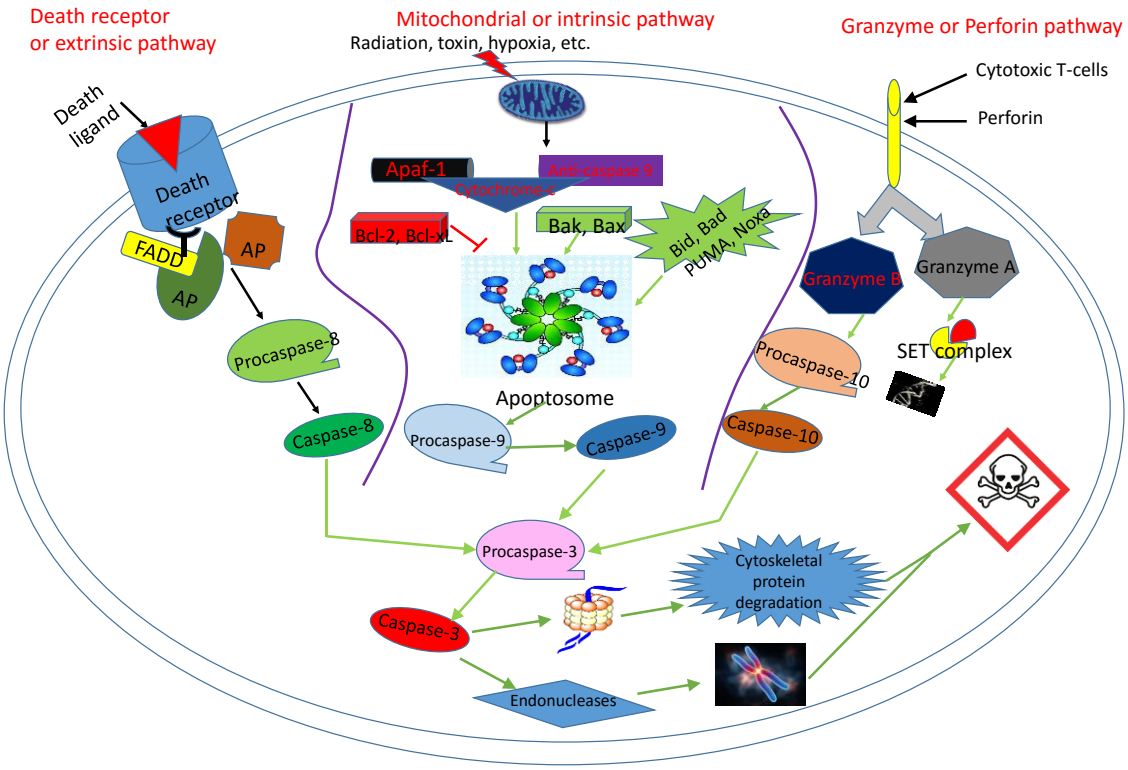


Fig 1-2 **Cell death pathways**.[36] Programmed cell death or apoptosis is the mechanism undergone by cells that are destined to die due to a variety of stimuli. There are three common pathways of programmed cell death viz. death receptor pathway, mitochondrial pathway and granzyme pathway.

1.5 Metal-complexes as functional antioxidant agents

Reactive oxygen species (ROS) have been proposed to cause oxidative damage in cells and are associted with a number of pathologies including cancer, aging and diabetes.[40; 41; 42] Metal complexes appear to be the ideal targets to confer antioxidant activity because they are able to exhibit multiple oxidation states and are able to form stable complexes with a variety of ligands, which are able to coordinate with different radicals or radical producing compounds or interact with the active sites of different

enzymes, thereby showing a variety of protective activities.[43] Recently, studies have demonstrated that complexes with Schiff's base ligands have the capability to scavenge ROS because of the presence of the C=N linkage, thus helping in the restoration of the redox balance in the damaged cells and organs.[44] The exact molecular signalling pathway is still elusive and hence needs much attention even though there are some studies indicating that these complexes affect the rate determining step of the superoxide dismutase cascade based on stop-flow or pulse radiolysis experiments. This incited research into aromatic and substituted ethylene diamine complexes to evaluate their antioxidant properties. Mn(III) complexes scavenge O^{2-}, H_2O_2 and lipid peroxides by coordinating to oxygen, resulting in the formation of several valence states which might be involved in scavenging ROS.[44]

1.6 Alcohol metabolism in the cells

The most common form of exogeneous alcohol is ethanol (CH_3CH_2OH), which is volatile, highly flammable and has a strong odor. Ethanol is known to cause neurotoxic effects.[45] It is not only neurotoxic, but affects almost every organ system of the body depending on the intake and the physical condition of the person consuming it. Depending on these factors, it might either have short term effects like drowsiness, nausea, headache, distorted vision, anemia and in extreme cases, may cause coma or long term effects like nerve damage, liver disease, high blood pressure, atherosclerosis, sexual problems etc.[46; 47] Reactive oxygen species, that are released during metabolic reactions, especially in the liver are capable of damaging biologically-important molecules like DNA and proteins.[48]

The cells have also evolved a few machineries to quench these radicals that are formed during metabolic processes and hence the radicals or the reactive species that are formed in excess are the ones that need attention, which when left untreated can damage the mitochondria.[49] Cytochrome P450 mixed-function oxidases use molecular oxygen while metabolizing xenobiotics and generate ROS, in addition to the species produced as a by product of the normal metabolic process.[50] In addition to this, immune cells also produce ROS, but are used to neuter the pathogens that invade the cell.

Alcohol is metabolized to acetaldehyde in the presence of the enzyme alcohol dehydrogenase and NAD^+. Acetaldehyde is then metabolized into acetate in the presence of several other alcohol dehydrogenases and results in the production of ROS as shown in Fig 1-3.[49; 51] The products of alcohol metabolism, ROS or acetaldehyde, are involved in a multitude of diseases and can cause DNA damage ranging from point mutations to gross chromosomal alterations. So, a method of quenching ROS produced in the cells is a major target for drug discovery.

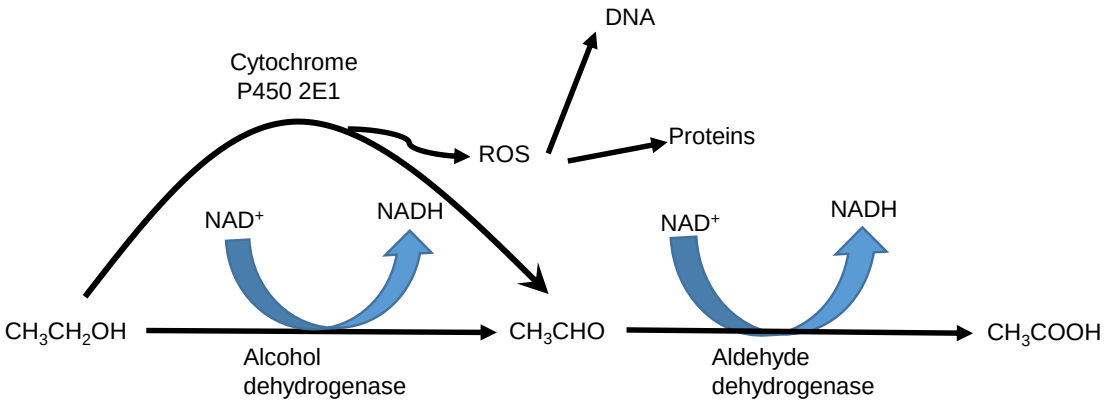


Fig 1-3 **Metabolism of alcohol in the hepatocytes (REF)**. Ethanol is metabolized in the liver by the cytochrome P450 2E1 or alcohol dehydrogenase in the presence of NAD^+. Metabolism by CYP450 2E1 generates reactive oxygen species (ROS) which can damage DNA and proteins and might result in a plethora of cellular malfunctions leading to pathological conditions. Acetaldehyde produced during the process is also known to cause DNA damage.

1.7 S-allyl cysteine

S-allyl cysteine is one of the components that is formed from a garlic preparation called Aged Garlic Extract (AGE) that involves the extraction using 25 % ethanol for more than 10 months, which leads to the enrichment of water-soluble cysteinyl moieties and reduces the harsh typical garlic odour in the solution.[52] Proper extraction method usually results in the production of S-allyl cysteine anywhere from 1.6-2.4 mg/kg dry weight of garlic.[53] The fact that the oral bioavailability of SAC fed at 100 mg/kg dose is 87-103 % in rats, mice and dogs makes it an interesting molecule.[54] The half-life of SAC in humans after oral administration was over 10 hours and the clearance time was over 30 hours. SAC is highly bioavailable in humans and it was shown that the total SAC content in the blood was about 450 μg.[55]

There are two reasons for including this molecule in this project. The first reason is because it is a small molecule containing a sulphur atom in it which can have physiologically relevant roles in the body. The second reason is because it is a widely studied compound in terms of its anti-microbial and anti-proliferative properties. A few studies have also linked it to the reduction of oxidative stress in cells but the molecular mechanism is still elusive.[56] So, we decided to evaluate its neuroprotective effects.

The anti-proliferative effects of S-allyl cysteine have been attributed to the aliphatic allyl group. Researchers predict that the allyl moiety of this compound is able to interact with the zinc atom in the active site of histone deacetylases and inhibit its activity resulting in the increased H3/H4 acetylation in cultured cancer cells such as DS19 and in rat liver at a slightly higher dosage.[57] Taking this as our foundation, we decided to synthesize a benzyl substituted cysteine instead of the allyl substituted cysteine and we studied the various effects of S-benzyl cysteine, in addition to S-allyl cysteine.

Chapter 2

ANTI-PROLIFERATIVE EFFECTS OF Mn(III) SALPHENS ON NEUROBLASTOMA CELL LINE SH-SY5Y

2.1 Introduction

Cancer is a major cause of death around the world. To combat with increasing rate of cancer associated mortality, there is always a need for the development of novel anticancer drugs with wide range of activities and high specificity for malignant cells and minimal side effects. Drugs with high specificity and relatively smaller size are the most coveted in the pharmaceutical research these days, since they reach the target tissues at a faster rate without any damage to the structure or with minimal alterations to the original structure, and hence they do not need any specially crafted transport system to deliver them to the target cells or tissues or organs.

Metallo-salen and metallo-salphen derivatives are a class of small molecule based drugs which have been shown to possess anti-neoplastic and antioxidant activities.[30] These compounds attracted our interest because of their relatively straight forward synthesis and their versatile mechanism of actions. Metallo-salens bind to and cleave the DNA and induce apoptosis in malignant cells.[32] These molecules induce apoptosis by increasing the oxidative stress in the tumor microenvironment. The advantage about these compounds is that their activity and selectivity towards malignant cells can be modulated by changing the functional groups and thereby lessening the adverse effects that might be caused by these compounds.

2.2 Materials and methods

2.2.1 General

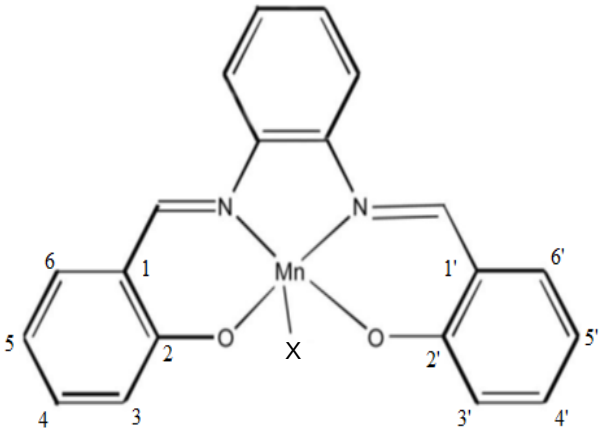
All the reagents for the synthesis and the buffers were purchased from Sigma-Aldrich unless noticed otherwise. The medium for cell culture DMEM (Dulbecco's Modified Eagle Medium), FBS (Fetal Bovine Serum), penicillin and streptomycin were purchased from Sigma-Aldrich. MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was purchased from Tokyo Chemical Industry Co. The cell lines used in this study, HT22 and SH-SY5Y were obtained from ATCC (American Type Culture Collection). Anhydrous Mn(III) acetate was bought from Spectrum Chemical Manufacturing Corporation. Fluostar-Omega, BMG, Labtech microplate reader was used to record the absorbance of 96-well plates following MTT assay.

2.2.2 Synthesis and characterization of Mn(III) salphens and their derivatives

Mn(III)-salphen and its derivatives were synthesized and characterized previously in our lab. Briefly, Mn(III) salphens were synthesized by mixing one equivalent of ortho-phenylenediamine with 2 equivalents of salicylaldehyde (for the synthesis of the parent salphen) or with 2 equivalents of salicylaldehyde derivatives in methanol and constantly stirring it over a 60 ˚C water bath for 15 minutes and left overnight to cool down. This yields precipitates of the respective salphens in a spectrum of colors ranging from yellow to orange. The precipitate was then washed with ice cold methanol and mixed with equivalent amounts of anhydrous Mn(III) acetate while heating it at 60 ˚C and stirring for 30 minutes, which results in a dark black/brown solution which was then cooled to room

temperature. The metallo-salphen derivatives were then precipitated out upon cooling after which they were filtered, washed with ice-cold methanol and dried.

The ligands were then characterized by ^{1}H-NMR and metal complexes were characterized by Electron-Spray Ionization-Mass spectroscopy (ESI-MS), Infrared (IR) spectroscopy and elemental analysis.[29; 30; 34; 58] For the cytotoxic assays and the clonogenic assay, the compounds were dissolved in cell culture grade DMSO. The structures of Mn(III) salphen derivatives and Mn(III) salnaphen derivatives are shown in Fig 2-1 and Fig 2-2 respectively (Mandal, et. al., (2012). U.S.Patent No.8198322. Austin, TX: U.S. Patent and Trademark Office).



4''= Cl	compound 1
4''= Br	compound 2
4''= NO_2	compound 3
4''= CH_3	compound 4
3,3'= OH	compound 5
4,4'= OH	compound 6
5,5'= OH	compound 7
3,3'= OCH_3	compound 8
4,4'= OCH_3	compound 9
X= OAc	

Fig 2-1 **Parent Mn(III) salphen**. The functional groups of the various compounds of interest in this study are represented in the right side of the image. 1 equivalent of ortho-phenylenediamine is mixed with two equivalents of salicylaldehyde in methanol at 60˚C for 15 min and left to cool down. Precipitate is washed with methanol and treated with an equivalent amount of Mn(III) acetate at 60˚C for 30 min and left to cool down to form a precipitate. Precipitate is washed with ice cold methanol, filtered, dried and characterized for purity.

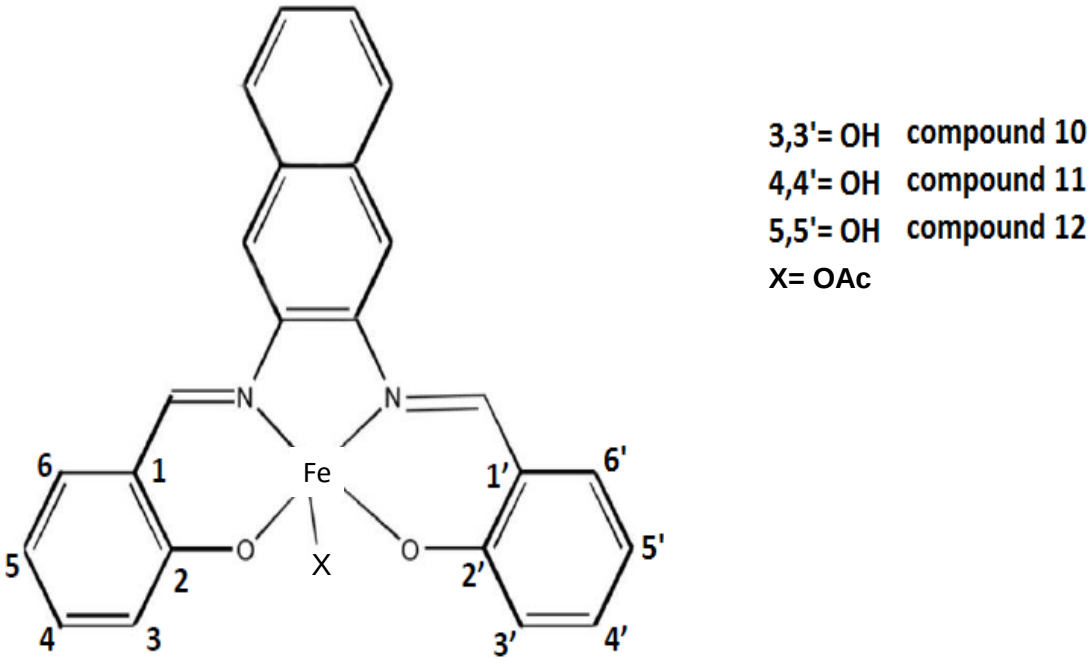


Fig 2-2 **Parent Fe(III) salphen**. The functional groups of the various compounds of interest in this study are represented in the right side of the image. 1 equivalent of ortho-napthylenediamine is mixed with two equivalents of salicylaldehyde in methanol at 60˚C for 15 min and left to cool down. Precipitate is washed with methanol and treated with an equivalent amount of Fe(III) acetate at 60˚C for 30 min and left to cool down to form a precipitate. Precipitate is washed with ice cold methanol, filtered, dried and characterized for purity.

2.3 Cell culture

Human neuroblastoma cell line (SH-SY5Y) and immortalized mouse hippocampal cell line (HT22) were grown as a monolayer and maintained in DMEM media supplemented with heat inactivated FBS (10 %), L-glutamine (1 %) and Penicillin/Streptomycin (1 %). Cells were grown in a humidity controlled incubator at 37 ˚C and 5 % CO_2. For the assays to determine the cytotoxicity of the compounds, the cells were grown in 96 well micro titre plates. Cells for the clonogenic assay were grown in 6-well plates.

2.4 MTT assay for the determination of IC_{50} value

MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the cytotoxicity of Mn(III) salphen and its derivatives. For the assay, approximately 10^4 cells were seeded into the 96 well micro titre plates (180 μL DMEM) and were allowed to grow until they were 50-70 % confluent. The cells were then treated with 20 μL of the compounds 1-12. Compounds 1-12 were dissolved in DMSO and the final concentration was maintained between the range 0-100 μM. The control cells were treated with the same amount of DMSO. The reactions were performed in 4 parallel replicates. The plates were then incubated at 37 ˚C, 5 % CO_2 for the next 48-72 hours after which the MTT assay was performed. 20 μL of 5 mg/ml MTT (Dissolved in PBS) was added into each of the 96 wells of the micro titre plate and incubated for 3-4 hours. During this incubation period, yellow coloured MTT is converted to purple coloured formazan crystals due to the action of the NAD(P)H-dependent cellular oxidoreductase enzymes present in the mitochondria of viable cells. The MTT solution is discarded and the formazan crystals are dissolved in DMSO and incubated for 45 minutes to 1 hour with continuous shaking. Then the absorbance is measured at 560 nm using a microplate reader (Fluostar-Omega, BMG Labtech). A graph was then plotted between the percent viable cells relative to control calculated using the absorbance values and the concentration, which is then used to determine the IC_{50} values of the compounds. Each experiment was repeated at least thrice.

2.5 Clonogenic assay

Approximately 10^6 cells were plated into nine 100 mm dish (three for control and the other six for treatments using compounds 5 and 9 (3plates each)) and was allowed to adhere to the plate and then incubated at 37 ˚C, 5 % CO_2 until they were 50-70 % confluent. Then the media was removed and the cells were washed twice with PBS and IC_{50} concentration of the compounds (5 and 9) were treated into the treatment plates and were incubated for 24 hours. Next day the cells were trypsinized and then approximately 500 cells were seeded on to each well of the six well plate. The cells were then incubated at 37 ˚C, 5 % CO_2 for 10-14 days after which the colonies were stained with crystal violet and counted under a microscope. A group of more than or equal to 50 cells was considered a colony. The experiment was conducted in triplicate.

2.6 Results

2.6.1 Analysis of the cytotoxicity of the Mn(III) salphens on SH-SY5Y and HT22 cell lines

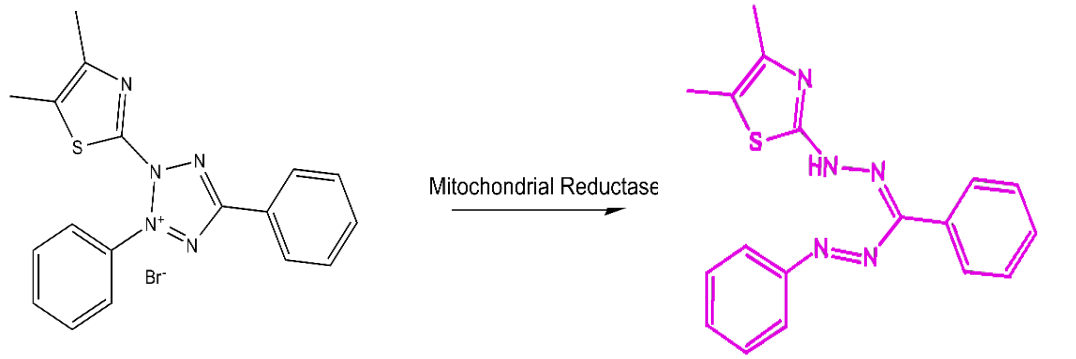
A total of 12 compounds (Fig 2-1 and 2-2) were analyzed for their cytotoxicity in neuroblastoma cell line SH-SY5Y and mouse hippocampal cell line HT22. The cytotoxicity of these compounds in human breast cancer cell line MCF7 and normal breast epithelial tissue MCF10 cell lines were evaluated previously in Dr. Mandal's lab[34] and the data are compared with the cytotoxicity in the SH-SY5Y and HT22 cell lines and is reported in the Table 2-1.

For the cytotoxicity assay, approximately 10^4 cells were seeded into the 96 well plates and incubated in a humidified CO_2 environment maintained at 37 ˚C for 12 hours. When the cells are 50-70 % confluent, the cells were treated with varying concentrations (0 – 100 μM) of each compound separately and then incubated for 96 hours and then subjected to MTT assay.

Table 2-1 The IC_{50} values of the compounds in MCF7, MCF10, SH-SY5Y and HT22 cell lines are tabulated in this table. * indicates that the IC_{50} values of the compounds in MCF7 and MCF10 were carried out by previous lab members and tabulated here from reference[34].

		IC50 in μM			
		MCF7*	MCF10*	SH-SY5Y	HT22
	Compounds				
Cpd-1	4"-Chloro Mn(III) Salphen			1.5 ± 0.5	59.02
Cpd-2	4"-Bromo Mn(III) Salphen			64 ± 2.1	>100
Cpd-3	4"-Nitro Mn(III) Salphen				>100
Cpd-4	4"-Methyl Mn(III) Salphen				97
Cpd-5	3,3'-dihydroxy Mn(III) Salphen	17 ± 0.4	52 ± 0.5	2.6 ± 0.8	>100
Cpd-6	4,4'-dihydroxy Mn(III) Salphen	> 100	> 100	> 100	>100
Cpd-7	5,5'-dihydroxy Mn(III) Salphen	45 ± 3.6	48 ± 4.3	35.5 ± 1.5	>100
Cpd-8	3,3'-dimethoxy Mn(III) Salphen	22 ± 0.8	28 ± 3.5	63.6 ± 1.9	>100
Cpd-9	4,4'-dimethoxy Mn(III) Salphen	15 ± 0.2	32 ± 0.7	9.8 + 1.6	>100
Cpd-10	3,3'-dihydroxy Fe(III) Salnaphen	0.2 ±0.06	>100	28.5 ± 1.2	0.342
Cpd-11	4,4'-dihydroxy Fe(III) Salnaphen	>100	>100	55 ± 0.8	>100
Cpd-12	5,5'-dihydroxy Fe(III) Salnaphen	> 100	> 100	> 100	>100

Upon addition of MTT, the NAD(P)H dependent oxidoreductases present in the mitochondria of the viable cells convert the MTT to formazan crystals as shown in the Scheme 2-1. The formazan crystals which emit a purple colour are monitored by measuring the absorbance at 560 nm using Fluostar-Omega microplate reader.



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

(*E*,*Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan **(Formazan)**

Scheme 2-1 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (Formazan) in the presence of NADPH dependent oxidoreductases present in the mitochondria of viable cells. This is both a qualitative and quantitative analysis which is used to identify the cytotoxicity of the drugs of interest.[58]

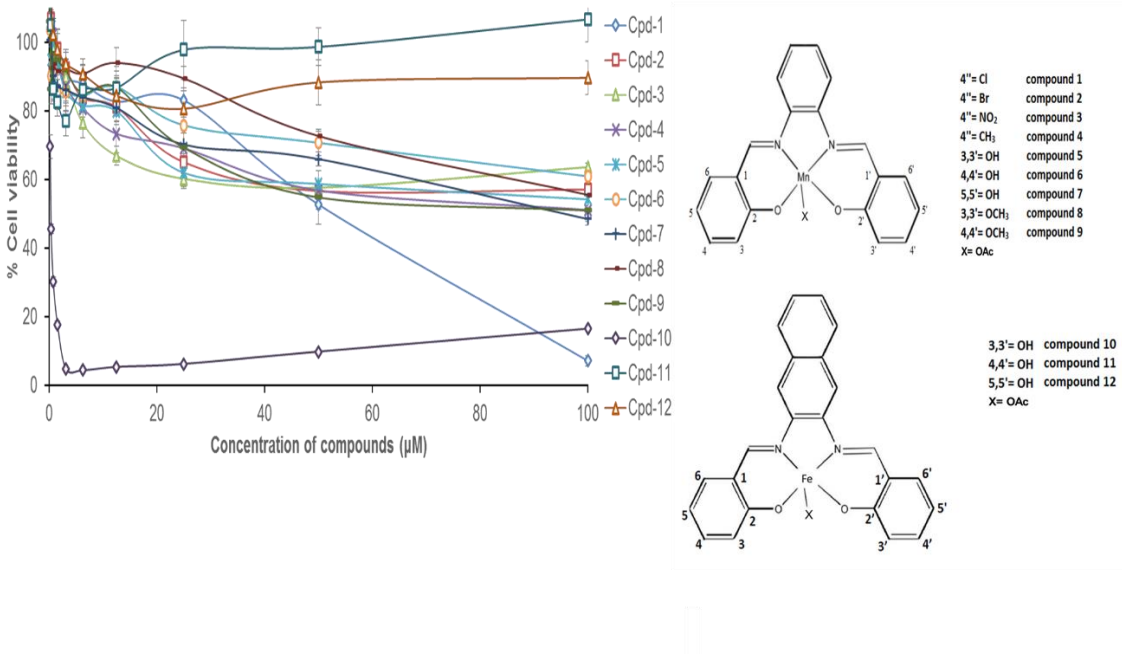


Fig 2-3 **IC_{50} graphs of the salphen and salnaphen derivatives in HT22 cell line**. The parent compound and the other compounds and their functional groups are shown in the right hand side of the graph. MTT assay – The cells are grown in a tissue culture dish until it reaches a 50-70 % confluency and then seeded into a 96-well plate in DMEM culture media. Once the confluency reaches 50-70 %, the cells are treated with 20 μL of the desired compound at various concentrations and incubated at 37 ˚C, 5 % CO_2 for 48-96 hours. Once the control wells are 100 % confluent, the wells are treated with 20 μL of MTT and incubated at 37 ˚C, 5 % CO_2 for 3-4 hours. Once the formazan crystals are formed, the crystals are dissolved in DMSO and then kept on a rocker for 1 hour and then the absorbance is measured using a spectrophotometer. Using the absorbance values, the percentage of viable cells is calculated.

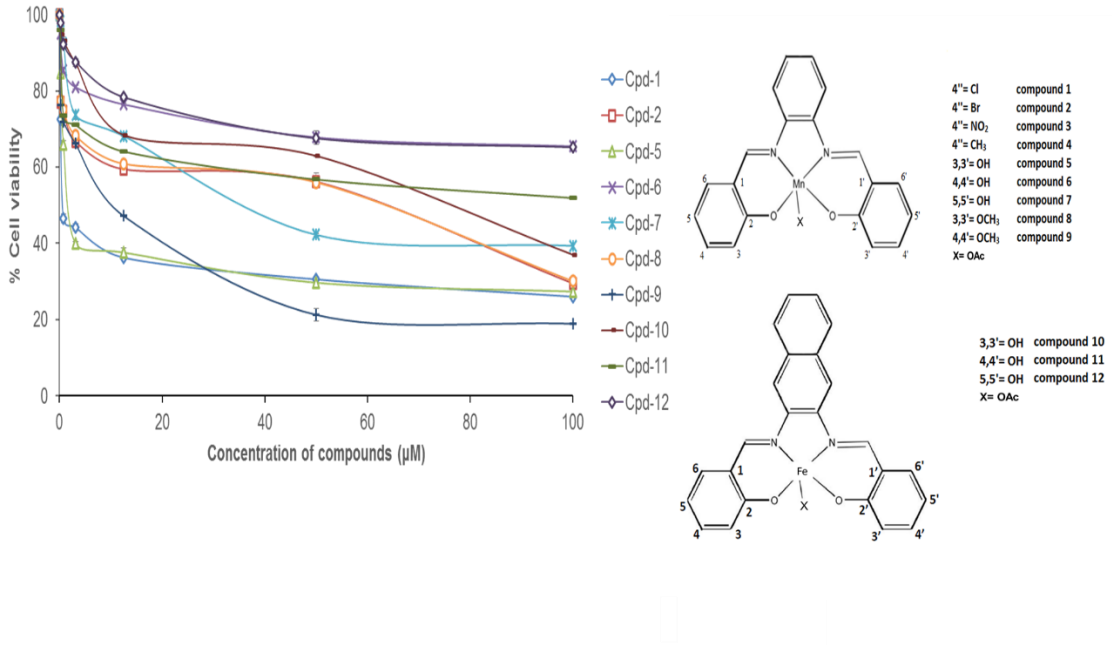


Fig 2-4 **IC_{50} graphs of the salphen and salnaphen derivatives in SH-SY5Y cell line**. The parent compound and the other compounds and their functional groups are shown in the right hand side of the graph. MTT assay – The cells are grown in a tissue culture dish until it reaches a 50-70 % confluency and then seeded into a 96-well plate in DMEM culture media. Once the confluency reaches 50-70 %, the cells are treated with 20 μL of the desired compound at various concentrations and incubated at 37 ˚C, 5 % CO_2 for 48-96 hours. Once the control wells are 100 % confluent, the wells are treated with 20 μL of MTT and incubated at 37 ˚C, 5 % CO_2 for 3-4 hours. Once the formazan crystals are formed, the crystals are dissolved in DMSO and then kept on a rocker for 1 hour and then the absorbance is measured using a spectrophotometer. Using the absorbance values, the percentage of viable cells is calculated.

The percentage of viable cells relative to control (either DMSO or PBS in the case of SAC) were calculated using the value of absorbance measured at λ=560 nm. The half maximal inhibitory concentration (IC_{50}) is measured by plotting the percentage viable cells against the concentration. Each experiment was repeated thrice with four parallel replicates. Fig. 2-4 shows the percentage of viable cells relative to control for the various compounds under study in HT22 cells; Fig 2-5 shows the percentage of viable cells

relative to control for the various compounds in SH-SY5Y cells. The compounds with the most activity are highlighted in red and are bold in Table 2-1 with their standard error of mean (SEM). IC50 of the compounds in the breast cancer cell line MCF-7 and normal breast epithelial cell line MCF10 were obtained from reference.[34] The cytotoxicity of the compounds in MCF7 and MCF10 cell lines were compared with the cytotoxicity obtained from this study on SH-SY5Y and HT22 cell lines. The compounds show a selective cytotoxicity towards the neuroblastoma cell line (SH-SY5Y) as compared to the normal hippocampal cell line (HT22) as it is evident from Table 2-1. Comparing this result with the results observed previously, it was found that change in the ethylene diamine bridge to an aromatic ring in the metallo-salen complex increased the cytotoxicity of these compounds on the neuroblastoma cell line. They corroborated their findings by microscopy using DAPI staining.[59] It was also seen that the methoxy-substituted derivatives in the salens had better cytotoxicity, but in the case of salphen compounds, that was not the case as both hydroxy-substituted and methoxy-substituted compounds showed good cytotoxic properties (shown in bold red in Table 2-1).

2.6.2 Inhibition of colony formation in SH-SY5Y cell line

From the table 2-1, it is clear that the compounds 3, 3'-dihydroxy Mn(III) salphen and 4, 4'-dimethoxy Mn(III) salphen are the most potent in the neuroblastoma cell line with an IC_{50} value of 2.6 ± 0.8 μM and 9.8 ± 1.6 μM respectively. So, these two compounds were tested for their ability to inhibit the formation of colonies in the SH-SY5Y cell line. Briefly, the cells were grown in tissue culture plates and once the cells

were 50-70 % confluent, the cells were treated with the IC_{50} concentration of 3, 3'-dihydroxy Mn(III) salphen and 4, 4'-dimethoxy Mn(III) salphen and were incubated at 37 ˚C, 5 % CO_2 for 20 hours. Then the cells were washed, trypsinized and counted under a microscope. Dilutions are made as necessary and the cells are plated into 6 well plates and then incubated at 37 ˚C, 5 % CO_2 for 10-14 days. Once the colonies are formed, the cells are washed twice with PBS and then fixed with 4 % formaldehyde and stained with crystal violet. The colonies are then counted under a microscope. A group of 50 cells is considered a colony.

As seen in Fig. 2-6 these two compounds (3, 3'-dihydroxy Mn(III) salphen and 4, 4'-dimethoxy Mn(III) salphen) effectively inhibit the formation of colonies. Fig. 2-6 shows the quantification data of the number of colonies formed in the controls and the treatment wells. The compound 3, 3'-dihydroxy Mn(III) salphen and 4, 4'-dimethoxy Mn(III) salphen showed an approximately 9-fold decrease in the number of colonies, compared to the control wells. These observations further demonstrate the anti-proliferative effects of metallo-salphens towards tumor cells.

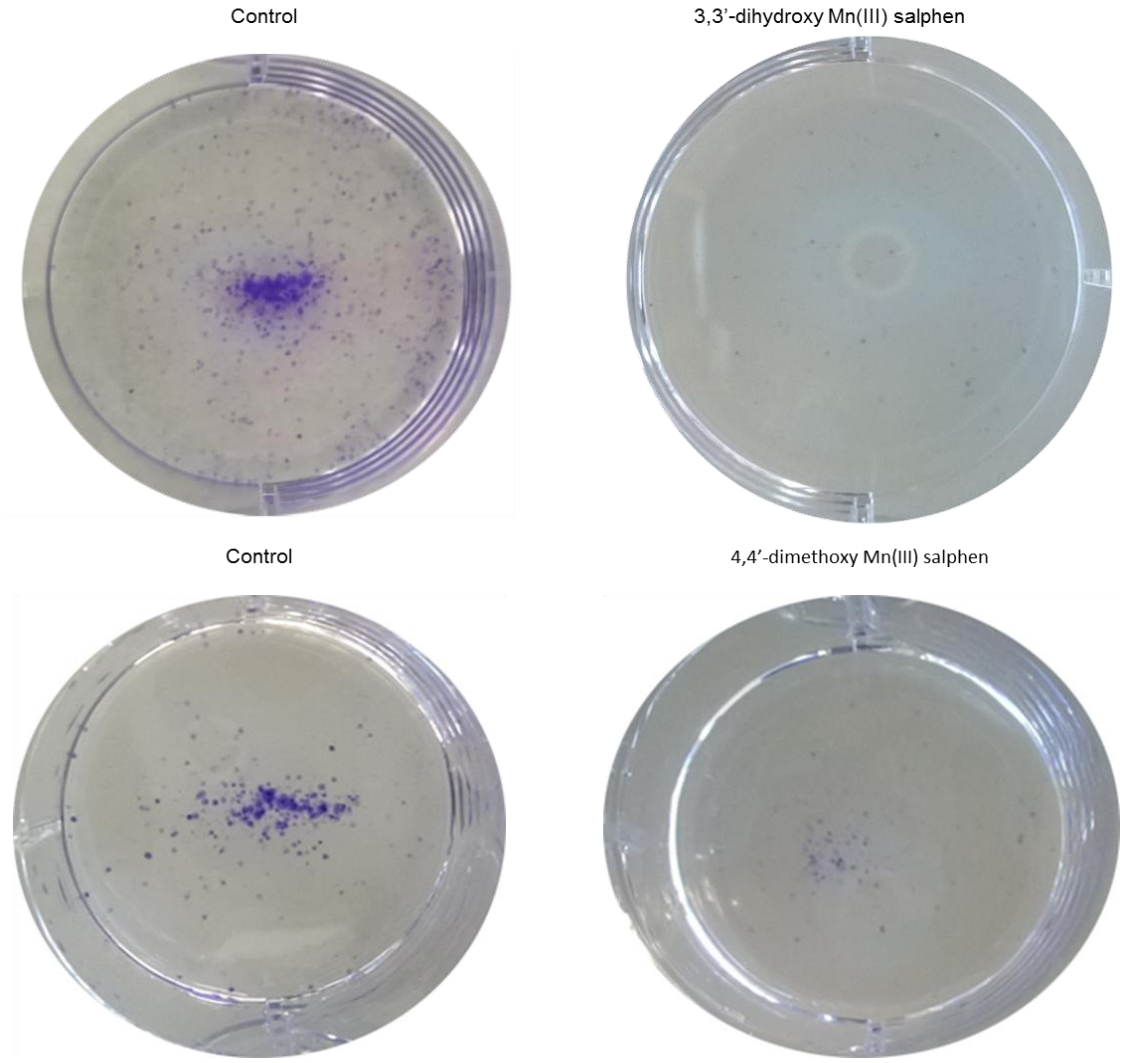


Fig 2-5 **Clonogenic assay of the compounds 3,3'-dihydroxy Mn(III) salphen and 4,4'-dimethoxy Mn(III) salphen in SH-SY5Y cell line**. The cells are grown in DMEM media till they are 50-70 % confluent. Then the cells are treated with the IC_{50} value of the respective compound for 20 hours and then washed twice with PBS and trypsinized and the cells are counted to seed the required number of cells are seeded into a 6 well plate and are incubated for 10-14 days at 37 ˚C, 5 % CO_2. Once the clear colonies start to form, the cells are washed with ice-cold PBS and then fixed with 4 % formaldehyde and stained with crystal violet. The colonies are then counted with a microscope and compared against the number of colonies formed in the control wells. The top row compares the control and the treatment with 3,3'-dihydroxy Mn(III) salphen and the bottom row compares the control and the treatment with 4,4'-dimethoxy Mn(III) salphen. It is clear from the image that the two compounds under consideration are effective in inhibiting the formation of colonies.

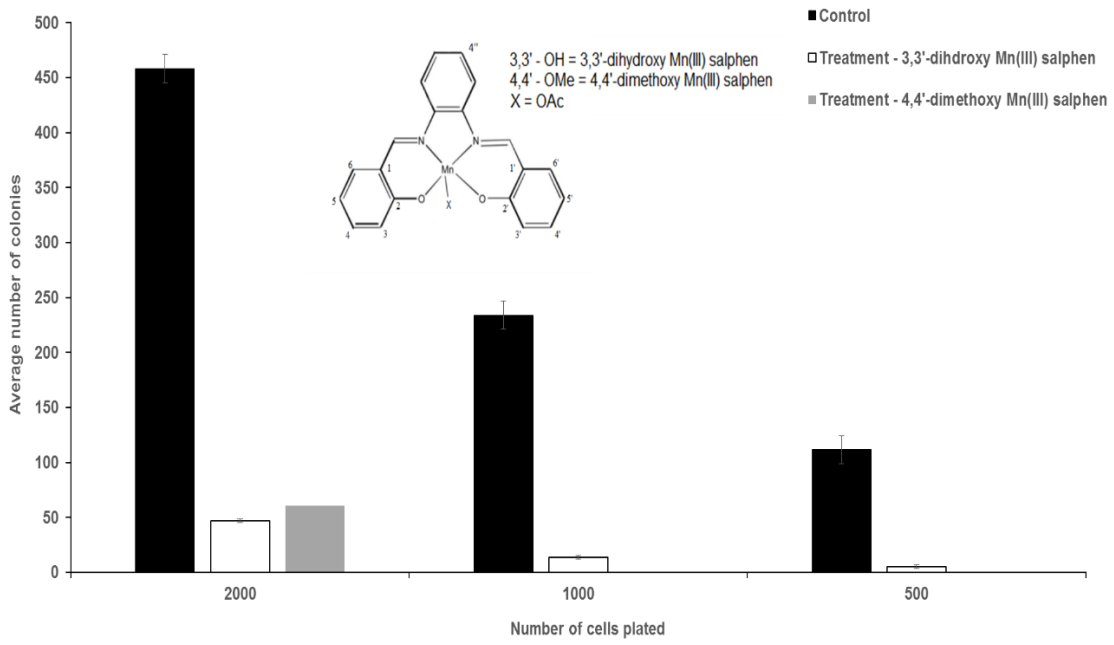


Fig 2-6 **Clonogenic assay of the compounds 3,3'-dihydroxy Mn(III) salphen and 4,4'-dimethoxy Mn(III) salphen in SH-SY5Y cell line.** The cells are grown in DMEM media till they are 50-70 % confluent. Then the cells are treated with the IC_{50} value of the respective compound for 20 hours and then washed twice with PBS and trypsinized and the cells are counted to seed the required number of cells are seeded into a 6 well plate and are incubated for 10-14 days at 37 ˚C, 5 % CO_2. Once the clear colonies start to form, the cells are washed with ice-cold PBS and then fixed with 4 % formaldehyde and stained with crystal violet. The colonies are then counted with a microscope and compared against the number of colonies formed in the control wells. The graph shows that when compared to control, there is a 9 fold decrease in the number of colonies formed in the treatment wells. It is clear from the graph that the two compounds under consideration are effective in inhibiting the formation of colonies.

2.7 Discussion

Continuing the work done by my previous lab colleagues, we analyzed the Mn(III) salphen compounds and the results shown here demonstrate that the metallo-salphen compounds have good cytotoxic effects on neuroblastoma cell line and were found to be protective in the case of hippocampal cell line. The previously hypothesized model for the cytotoxic effect of these compounds is by the release of cytochrome-c from the mitochondria, which results in the activation of the apoptotic cascade resulting in the release of the caspases [29; 31; 34], but the exact pathway for the activity observed in this study is still not understood. Even though the mechanism is not still understood, the results show that these compounds are potential chemotherapeutic drugs, if in vivo studies are successful and the mechanism of action of action is traced out.

Chapter 3

ANALYSIS OF ANTIOXIDANT PROPERTIES OF Mn(III) SALPHENS, S-ALLYL CYSTEINE AND S-BENZYL CYSTEINE

3.1 Introduction

The current chemotherapeutic drugs are involved in the production of a lot of free radicals which are involved in causing a plethora of side effects and hence there is a need to discover drugs which have antioxidant properties along with the ability to selectively target the malignant cells. Metal complexes are better candidates because they show multiple oxidation states which helps in co-ordinating with various ligands (reactive oxygen species like superoxide anion, hydroxyl radical, peroxynitrite etc.) and quench these radicals which otherwise might damage the nearby cells.[6; 60] Recent discoveries have shown that metal complexes with Schiff's base ligands have the ability to scavenge ROS and restoring the redox balance in the damaged cells and tissues.[44] This is a striking discovery since the preparation of Schiff's base ligands is very straightforward and they have the ability to stabilize different metal ions in various oxidation states by controlling the activity of the metals in various organ systems of the body. The C=N link confers the Schiff's base ligands its biological activity and the anti-oxidant properties.[11] Since these complexes resemble the active site of the endogenous antioxidant enzymes in the body, they show antioxidant activities. Many studies indicate that metal complexes with Schiff's base show anti-arthritic, analgesic and anti-tumor activities. Mn(III) complexes are also studied for their ability to scavenge O_2^-, H_2O_2 and lipid peroxides.

The compounds under investigation in this study have a manganese coordinated to oxygen and nitrogen which leads to several possible oxidation states for the central metal atom, which is predicted to be the important reason for these compounds' antioxidant properties. Therefore, we designed and screened various metallo-salphen complexes for their ability to quench the reactive oxygen species produced upon addition of alcohol. To test the ability of these complexes to quench the ROS, we used an ethanol-based antioxidant assay. Alcohol is metabolized by the alcohol dehydrogenase enzyme and/or cytochrome P450 2E1 which converts ethanol into acetaldehyde, during which they release oxygen, superoxide and peroxide ions. The acetaldehyde produced in this process was found to promote the growth of cancer by intervening in the DNA replication machinery. It was also found that, the acetaldehyde produced can cause point mutations to gross chromosomal alteration leading to a wide range of disorders. This initiates replication errors and mutations in the tumor suppressing genes.[50]

In addition to the metal complexes, we also evaluated the antioxidant activity of a natural compound, S-allyl cysteine obtained from aged garlic extract. The compound S-allyl cysteine has been shown to have antioxidant activities, but the mechanism is still elusive and hence needs further clarification.[61] To test the antioxidant property of this compound, we synthesized pure S-allyl cysteine in collaboration with Dr. Alejandro Bugarin (University of Texas at Arlington). We also synthesized a benzyl derivative of cysteine in collaboration with Dr. Alejandro Bugarin to evaluate the importance of the allyl group in S-allyl cysteine in conferring the antioxidant property.

3.2 Materials and methods

All the reagents needed for the synthesis of the compounds and buffers were procured from Sigma-Aldrich unless otherwise indicated. The medium for tissue culture, DMEM (Dulbecoo's Modified Eagle Medium) and F12 media, FBS (Fetal Bovine Serum), penicillin and streptomycin were purchased from Sigma-Aldrich. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was purchased from Tokyo Chemical Industry Co. The cell line for this study HT22 was obtained from ATCC (American Type Culture Collection). Fluostar-Omega, BMG, Labtech microplate reader was used to record the value of absorbance for the 96 well micro titre plate after performing the MTT assay.

3.2.1 Synthesis of S-allyl cysteine and S-benzyl cysteine

S-allyl cysteine is a major compound that is formed during the aging process of garlic.[61] Briefly, garlic cloves are freshly cut and soaked into a 25 % ethanol solution and left to stand at room temperature for at least 10 months and then the liquid alone is filtered using a syringe filter and then evaporated to give S-allyl cysteine in powder form. Here we have synthesized S-allyl cysteine and S-benzyl cysteine.

L-cysteine is mixed with 1.1 equivalents of allyl bromide in the presence of EtONa and EtOH at 25 ˚C. This yields a white precipitate of S-allyl cysteine (76 % yield) which is filtered, then dried under vacuum and is characterized using ^{1}H-NMR.[62]

L-cysteine is mixed with 1.1 equivalents of benzyl bromide in the presence of NaOH and EtOH at 25˚C. This yields a white precipitate of S-benzyl cysteine (94 %

yield) which is filtered, then dried under vacuum and is characterized by ^{1}H-NMR.[63] These compounds were synthesized in collaboration with Dr. Alejandro Bugarin (University of Texas at Arlington). The structures of these two compounds are shown in Fig. 3-1.

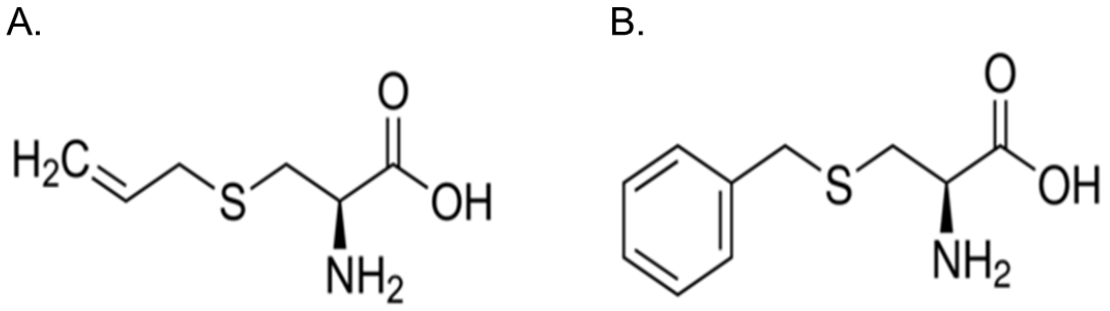


Fig 3-1 **Structure of A. S-Allyl Cysteine (SAC) and B. S-Benzyl Cysteine (SBC).** 1 equivalent of L-cysteine is mixed with 1.1 equivalents of allyl bromide in the presence of EtONa and EtOH at 25˚C and stirred for 20 hours results in the production of S-allyl cysteine (76% yield). 1 equivalent of R-cysteine is mixed with 1 equivalents of benzyl bromide in the presence of NaOH and EtOH at 25˚C and stirred for 20 hours results in the production of S-benzyl cysteine (94% yield). Both the products are obtained as white precipitates.

3.3 Titration to determine the optimum ethanol concentration

To identify the optimum concentration at which ethanol showed toxic effects, MTT assay was performed using ethanol treatment on the cells with a concentration range from 0-5000mM. Approximately 10^4 cells were seeded into the 96 well micro titre plate and were grown till they were 50-70 % confluent and then the cells were treated with ethanol with various concentrations for 24-36 hours. Control cells were treated with just the complete media. Once ethanol was added to the cells, the plate was covered with parafilm to prevent the evaporation of the highly volatile ethanol. Each reaction was

performed in 4 parallel replicates and was carried out thrice. After 24-36 hours of the treatment, MTT assay was performed by adding 20 μL of MTT solution into each well of the micro titre plate and incubated for 3-4 hours during which the cellular oxidoreductases convert MTT into purple coloured formazan crystals. The media is then discarded and the crystals are dissolved in DMSO and incubated for 45 minutes to 1 hour and is kept on a rocker. Then the absorbance is measured at 560 nm using the Fluostar-Omega, BMG, Labtech microplate reader. Using the absorbance values, the percentage of viable cells is calculated and then it is plotted against the concentration of the compounds to obtain the IC_{50} values.

3.4 Antioxidant properties of Mn(III) salphens, SAC and SBC under oxidative stress

80 μL of DMEM containing approximately 5000 cells were seeded into the 96 well micro titre plate and were incubated at 37 ˚C, 5 % CO_2 until they reach a confluency of 50-70 %. The cells are then treated with 20 μL of various concentrations of the compounds under investigation. 100 μL of DMEM 1000 mM of ethanol is then added into the wells bringing the final concentration of ethanol in the wells to 500 mM. The control cells were treated with the same amount of DMSO and the ethanol controls had 120 μL of 500 mM ethanol. After 20 hours of incubation, MTT assay was performed and then the absorbance was measured at 560 nm using Fluostar-Omega, BMG, Labtech. A bar graph was plotted to compare the cell viability of the ethanol control cells and the cells treated with the drug at different concentrations.

3.5 Results

3.5.1 Evaluation of the cytotoxicity of Ethanol in HT22 and SH-SY5Y cell lines

To optimize the concentration of ethanol for analysing the oxidative stress condition, cytotoxicity assay was performed. A range of ethanol concentrations (100-5000 mM) was analysed in HT22 cells and each of these experiments were conducted in 4 parallel replicates and was repeated thrice. The plates were covered with a parafilm immediately after addition of the appropriate concentration of ethanol to inhibit the evaporation of ethanol. 24-36 hours post-treatment, the cell viability was calculated using the MTT assay.

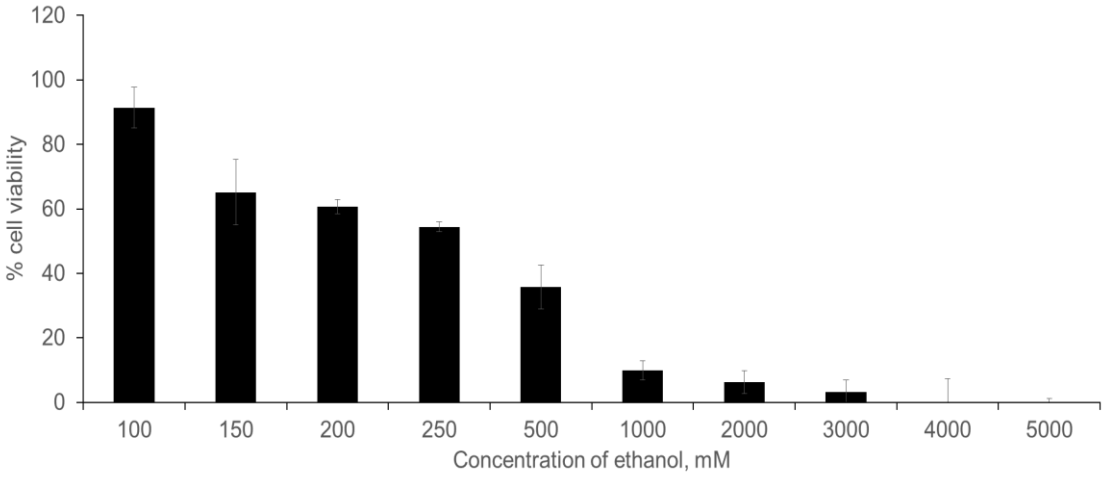


Fig 3-2 Titration of ethanol to find the optimum concentration necessary for evaluating the antioxidant activity of the compounds of interest in HT22 cell line. MTT assay – The cells are grown in a tissue culture dish until it reaches a 50-70 % confluency and then seeded into a 96-well plate in DMEM culture media. Once the confluency reaches 50-70 %, the cells are treated with 20 µL of varying concentrations of ethanol and incubated at 37 ˚C, 5 % CO_2 for 48-96 hours. Once the control wells are 100 % confluent, the wells are treated with 20 µL of MTT and incubated at 37 ˚C, 5 % CO_2 for 3-4 hours. Once the formazan crystals are formed, the crystals are dissolved in DMSO and then kept on a rocker for 1 hour and then the absorbance is measured using a spectrophotometer. Using the absorbance values, the percentage of viable cells is calculated. This experiment was performed by Dr. Arunoday Bhan in Dr. Mandal's lab.

Using the absorbance value, the percentage of viable cells relative to control were calculated and was then plotted as a function of concentration to determine the IC_{50} values. Fig. 3-2 shows the percentage of viable cells relative to control in HT22 cell line. The IC_{50} value for ethanol in HT22 was calculated to be 335±2.1 mM. This was the concentration of ethanol used for the ethanol-based antioxidant assay to determine the anti-oxidant properties of Mn(III) salphen derivatives, SAC and SBC.

3.5.2 Antioxidant properties of Mn(III) salphens under ethanol-induced oxidative stress

For the determination of the antioxidant properties of the compounds, 80 μL of DMEM containing approximately 5000 cells were seeded into a 96 well micro titre plate and were incubated till they reached a confluency of 50-70 %. The cells were then treated with 20 μL of the compounds showing good cytotoxic effects (Compounds 5-9) in a range of concentrations (0-100 μM). 100 μL of DMEM containing 1000 μM ethanol was added to the wells so that the final concentration of ethanol in the cells was 500 mM and the cells were incubated for 24 hours at 37 ˚C, 5 % CO_2. Control cells were treated with the same amount of DMSO, while the ethanol controls were treated with same amount of ethanol without the addition of any drug. Since ethanol was used, the micro titre plates were covered with parafilm to inhibit the evaporation of ethanol. After 24 hours of treatment, the cell viability was calculated using the MTT assay.

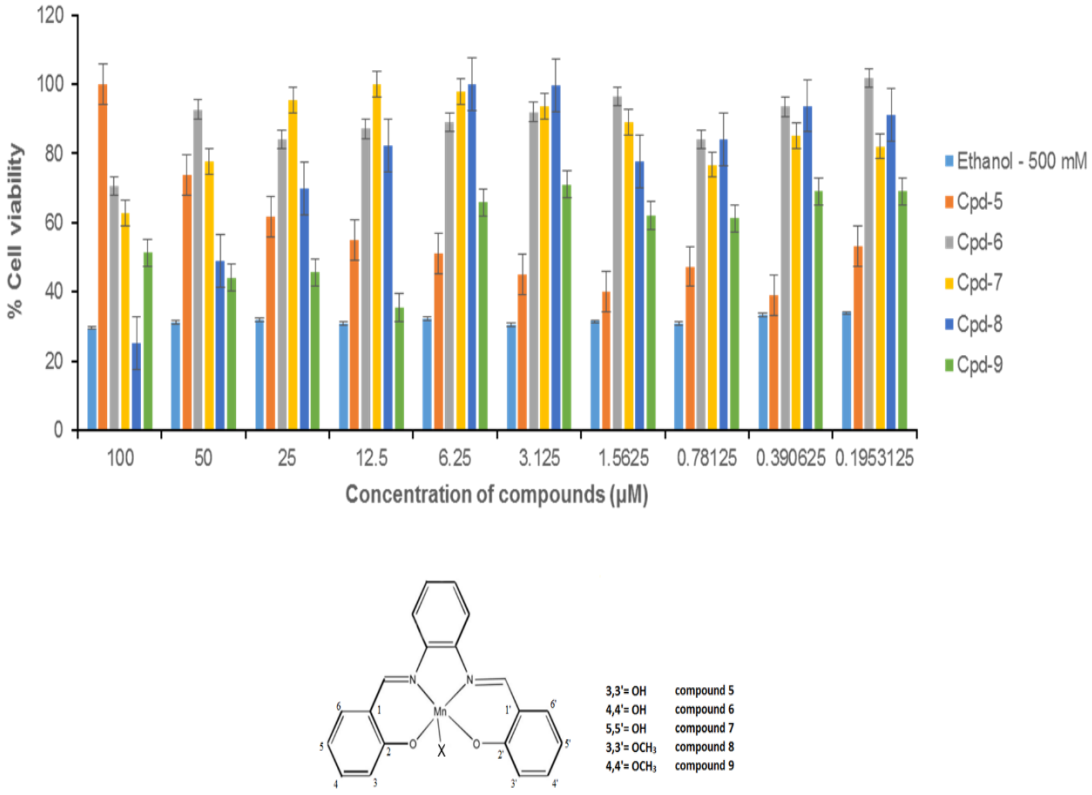


Fig 3-3 **Antioxidant activity of metallo-salphen compounds in HT22 cell line**. The parent compound and the other compounds and their functional groups are shown in the right hand side of the graph. MTT assay – The cells are grown in a tissue culture dish until it reaches a 50-70 % confluency and then seeded into a 96-well plate in DMEM culture media. Once the confluency reaches 50-70 %, the cells are treated with 100 µL of 500 mM ethanol dissolved in DMEM to start the release of reactive oxygen species. The cells are then treated with 20 µL of the desired compound at various concentrations and incubated at 37 ˚C, 5 % CO_2 for 20 hours. Once the control wells are 100 % confluent, the wells are treated with 20 µL of MTT and incubated at 37 ˚C, 5 % CO_2 for 3-4 hours. Once the formazan crystals are formed, the crystals are dissolved in DMSO and then kept on a rocker for 1 hour and then the absorbance is measured using a spectrophotometer. Using the absorbance values, the percentage of viable cells is calculated.

The percentage of viable cells was calculated using the absorbance values and were then plotted against the concentration of the compounds relative to ethanol concentration. Fig 3-3 shows that the viability of the HT22 cells increases with increase

in concentration of the compound 3,3'-dihydroxy Mn(III) salphen, remains the same irrespective of the concentration of 4,4'-dihydroxy Mn(III) salphen, shows an irregular trend in the case of 5,5'-dihydroxy Mn(III) salphen and 3,3'-dimethoxy Mn(III) salphen. In the case of 4,4'-dimethoxy Mn(III) salphen, the HT22 cell viability is maintained the same until the concentration reaches 12.5 μM and then decreases to a certain value and then remains the same thereafter. It can be concluded that the metallo-salphen complexes have significant antioxidant properties in HT22 cell line upon an ethanol insult.

3.5.3 Antioxidant properties of SAC and SBC under ethanol-induced oxidative stress

For the determination of the antioxidant properties of the compounds, 80 μL of DMEM containing approximately 5000 cells were seeded into a 96 well micro titre plate and were incubated till they reached a confluency of 50-70 %. The cells were then treated with 20 μL of the compounds showing good cytotoxic effects (Compounds 5-9) in a range of concentrations (0-100 μM). 100 μL of DMEM containing 1000 μM ethanol was added to the wells so that the final concentration of ethanol in the cells was 500 mM and the cells were incubated for 24 hours at 37 ˚C, 5 % CO_2. Control cells were treated with the same amount of DMSO, while the ethanol controls were treated with same amount of ethanol without the addition of any drug. Since ethanol was used, the micro titre plates were covered with parafilm to inhibit the evaporation of ethanol. After 24 hours of treatment, the cell viability was calculated using the MTT assay. The percentage of viable

cells was calculated using the absorbance values and were then plotted against the concentration of the compounds relative to ethanol concentration.

SAC and SBC were evaluated for their antioxidant properties because many studies indicate that SAC has antioxidant properties in neuronal cell lines.[64]

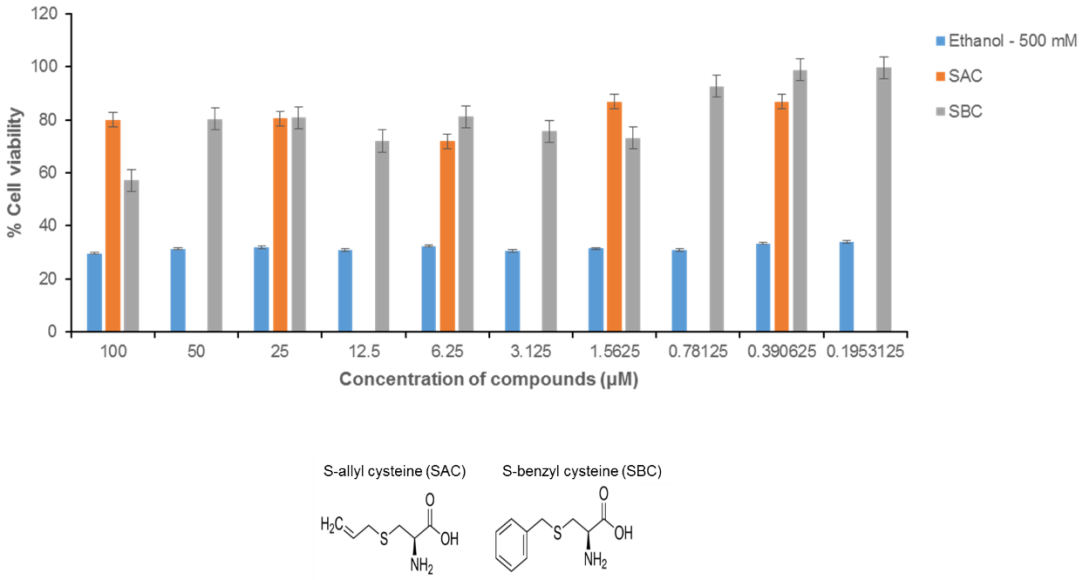


Fig 3-4 **Antioxidant activity of the SAC and SBC in HT22 cell line**. The parent compound and the other compounds and their functional groups are shown in the right hand side of the graph. MTT assay – The cells are grown in a tissue culture dish until it reaches a 50-70 % confluency and then seeded into a 96-well plate in DMEM culture media. Once the confluency reaches 50-70 %, the cells are treated with 100 µL of 500 mM ethanol dissolved in DMEM to start the release of reactive oxygen species. The cells are then treated with 20 µL of the desired compound at various concentrations and incubated at 37 ˚C, 5 % CO_2 for 20 hours. Once the control wells are 100 % confluent, the wells are treated with 20 µL of MTT and incubated at 37 ˚C, 5 % CO_2 for 3-4 hours. Once the formazan crystals are formed, the crystals are dissolved in DMSO and then kept on a rocker for 1 hour and then the absorbance is measured using a spectrophotometer. Using the absorbance values, the percentage of viable cells is calculated.

Fig 3-4 depicts the antioxidant properties of SAC and SBC. SAC is a special case where the HT22 cell viability remains the same starting from a lower range up to 600 μM (Data shown only up to 100 μM). S-benzyl cysteine showed a good trend till the concentration was 50 μM, but an increase in concentration resulted in a decrease in the cell viability. It can be concluded that the natural compound SAC and its synthetic analogue SBC have significant antioxidant properties in HT22 cell line upon an ethanol insult.

3.6 Discussion

The metallo-salphen complexes (compounds 5-9) showed a protective effect on the HT22 cell line upon induction of oxidative stress by ethanol. Previously it was also shown to have selective toxicity towards the neuroblastoma cell line SH-SY5Y (data not shown).[59] The compounds were also shown to have less toxicity towards the HT22 cells in the presence of ethanol and also showed an enhanced cell growth, whereas they were highly toxic in the SH-SY5Y cell line. These results inclines us towards the conclusion that these compounds are potential chemotherapeutic drugs and novel antioxidants. S-benzyl cysteine did not show any enhanced protective effects over S-allyl cysteine, which demonstrates that the aromatic group is not important in this case, unlike the metallo-complexes. With these results as a foundation, the compounds need further investigation in *in vivo* models.

3.7 Summary and Conclusion

With the development of chemotherapy resistance in patients suffering from cancer, there is a huge demand for designing new chemotherapeutic drugs which can either overcome the resistance or which can take an alternate route to destroy or neuter the malignant cells. Even though many compounds are synthesized and screened for their biological activity, only a handful of them go into clinical trials and it takes around a decade to be accessible in routine practice. The currently prescribed drugs that are in practice are associated with a lot of side-effects and are limited to certain types of cancers. This raises the bar for drug developers as it is difficult to determine the physicochemical interactions of these compounds in the body and the rise of pharmacogenomics makes the problem even more complicated.

Biochemical evaluation of a drug is needed to understand the pathway the drug follows to perform the intended function so that the molecule can be tweaked in order to get the desired function. In this study, we performed cytotoxic analysis and antioxidant properties of 14 different compounds and narrowed it down to 2 compounds which may have potential therapeutic property. 5 compounds were selectively toxic towards SH-SY5Y cells and the most potent compounds were tested for their activity using clonogenic assay to determine their ability to inhibit the formation of colonies. The compounds 5-9, SAC and SBC showed good protective effects on the HT22 cell line upon induction of oxidative stress by ethanol.

References

1. Riordan, J. F. (1977). The role of metals in enzyme activity. *Ann Clin Lab Sci* **7**, 119-29.
2. Vallee, B. L., Rupley, J. A., Coombs, T. L. & Neurath, H. (1958). THE RELEASE OF ZINC FROM CARBOXYPEPTIDASE AND ITS REPLACEMENT. *Journal of the American Chemical Society* **80**, 4750-4751.
3. Auld, D. S. & Bergman, T. (2008). Medium- and short-chain dehydrogenase/reductase gene and protein families : The role of zinc for alcohol dehydrogenase structure and function. *Cell Mol Life Sci* **65**, 3961-70.
4. Turel, I. & Kljun, J. (2011). Interactions of metal ions with DNA, its constituents and derivatives, which may be relevant for anticancer research. *Curr Top Med Chem* **11**, 2661-87.
5. Che, C. M. & Siu, F. M. (2010). Metal complexes in medicine with a focus on enzyme inhibition. *Curr Opin Chem Biol* **14**, 255-61.
6. Desoize, B. (2004). Metals and metal compounds in cancer treatment. *Anticancer Res* **24**, 1529-44.
7. Mezencev, R. (2015). Interactions of cisplatin with non-DNA targets and their influence on anticancer activity and drug toxicity: the complex world of the platinum complex. *Curr Cancer Drug Targets* **14**, 794-816.
8. Peyrone, M. (1844). Ueber die Einwirkung des Ammoniaks auf Platinchlorür. *Justus Liebigs Annalen der Chemie* **51**, 1-29.
9. Rosenberg, B., Vancamp, L. & Krigas, T. (1965). INHIBITION OF CELL DIVISION IN ESCHERICHIA COLI BY ELECTROLYSIS PRODUCTS FROM A PLATINUM ELECTRODE. *Nature* **205**, 698-9.
10. Arriagada, R., Bergman, B., Dunant, A., Le Chevalier, T., Pignon, J. P. & Vansteenkiste, J. (2004). Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *N Engl J Med* **350**, 351-60.
11. Kelland, L. (2007). The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* **7**, 573-84.
12. Wheate, N. J., Walker, S., Craig, G. E. & Oun, R. (2010). The status of platinum anticancer drugs in the clinic and in clinical trials. *Dalton Trans* **39**, 8113-27.
13. Hannon Michael, J. (2007). Metal-based anticancer drugs: From a past anchored in platinum chemistry to a post-genomic future of diverse chemistry and biology. In *Pure and Applied Chemistry*, Vol. 79, pp. 2243.
14. Gasser, G., Ott, I. & Metzler-Nolte, N. (2011). Organometallic Anticancer Compounds. *J Med Chem* **54**, 3-25.

15. Esteban-Fernandez, D., Moreno-Gordaliza, E., Canas, B., Antonia Palacios, M. & Milagros Gomez-Gomez, M. (2010). Analytical methodologies for metallomics studies of antitumor Pt-containing drugs. *Metallomics* **2**, 19-38.
16. Heffern, M. C., Yamamoto, N., Holbrook, R. J., Eckermann, A. L. & Meade, T. J. (2013). Cobalt derivatives as promising therapeutic agents. *Curr Opin Chem Biol* **17**, 189-96.
17. Kopf-Maier, P., Kopf, H. & Neuse, E. W. (1984). Ferricenium complexes: a new type of water-soluble antitumor agent. *J Cancer Res Clin Oncol* **108**, 336-40.
18. Indumathy, R., Radhika, S., Kanthimathi, M., Weyhermuller, T. & Unni Nair, B. (2007). Cobalt complexes of terpyridine ligand: crystal structure and photocleavage of DNA. *J Inorg Biochem* **101**, 434-43.
19. Ott, I., Schmidt, K., Kircher, B., Schumacher, P., Wiglenda, T. & Gust, R. (2005). Antitumor-active cobalt-alkyne complexes derived from acetylsalicylic acid: studies on the mode of drug action. *J Med Chem* **48**, 622-9.
20. Messori, L., Marcon, G. & Orioli, P. (2003). Gold(III) Compounds as New Family of Anticancer Drugs. *Bioinorg Chem Appl* **1**, 177-87.
21. Tiekink, E. R. T. (2002). Gold derivatives for the treatment of cancer. *Critical Reviews in Oncology/Hematology* **42**, 225-248.
22. Caruso, F. & Rossi, M. (2004). Antitumor titanium compounds and related metallocenes. *Met Ions Biol Syst* **42**, 353-84.
23. Harding, M. M. & Mokdsi, G. (2000). Antitumour metallocenes: structure-activity studies and interactions with biomolecules. *Curr Med Chem* **7**, 1289-303.
24. Rademaker-Lakhai, J. M., van den Bongard, D., Pluim, D., Beijnen, J. H. & Schellens, J. H. (2004). A Phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. *Clin Cancer Res* **10**, 3717-27.
25. Pluim, D., van Waardenburg, R. C., Beijnen, J. H. & Schellens, J. H. (2004). Cytotoxicity of the organic ruthenium anticancer drug Nami-A is correlated with DNA binding in four different human tumor cell lines. *Cancer Chemother Pharmacol* **54**, 71-8.
26. Bergamo, A., Gava, B., Alessio, E., Mestroni, G., Serli, B., Cocchietto, M., Zorzet, S. & Sava, G. (2002). Ruthenium-based NAMI-A type complexes with in vivo selective metastasis reduction and in vitro invasion inhibition unrelated to cell cytotoxicity. *Int J Oncol* **21**, 1331-8.
27. Collery, P., Keppler, B., Madoulet, C. & Desoize, B. (2002). Gallium in cancer treatment. *Crit Rev Oncol Hematol* **42**, 283-96.

28. Ott, I. & Gust, R. (2007). Preclinical and clinical studies on the use of platinum complexes for breast cancer treatment. *Anticancer Agents Med Chem* **7**, 95-110.
29. Ansari, K. I., Grant, J. D., Woldemariam, G. A., Kasiri, S. & Mandal, S. S. (2009). Iron(III)-salen complexes with less DNA cleavage activity exhibit more efficient apoptosis in MCF7 cells. *Org Biomol Chem* **7**, 926-32.
30. Ansari, K. I., Kasiri, S., Grant, J. D. & Mandal, S. S. (2011). Fe(III)-salen and salphen complexes induce caspase activation and apoptosis in human cells. *J Biomol Screen* **16**, 26-35.
31. Mandal, S. S. (2011). Metallo-salen complexes show promise towards treatment of leukemia. *Leuk Res* **35**, 571-2.
32. Mandal, S. S., Kumar, N. V., Varshney, U. & Bhattacharya, S. (1996). Metal-ion-dependent oxidative DNA cleavage by transition metal complexes of a new water-soluble salen derivative. *J Inorg Biochem* **63**, 265-72.
33. Routier, S., Vezin, H., Lamour, E., Bernier, J. L., Catteau, J. P. & Bailly, C. (1999). DNA cleavage by hydroxy-salicylidene-ethylendiamine-iron complexes. *Nucleic Acids Res* **27**, 4160-6.
34. Ansari, K. I., Grant, J. D., Kasiri, S., Woldemariam, G., Shrestha, B. & Mandal, S. S. (2009). Manganese(III)-salens induce tumor selective apoptosis in human cells. *J Inorg Biochem* **103**, 818-26.
35. Ott, I. & Gust, R. (2007). Non platinum metal complexes as anti-cancer drugs. *Arch Pharm (Weinheim)* **340**, 117-26.
36. Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516.
37. Trapani, J. A. & Smyth, M. J. (2002). Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* **2**, 735-47.
38. Sakahira, H., Enari, M. & Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391**, 96-9.
39. Fan, Z., Beresford, P. J., Oh, D. Y., Zhang, D. & Lieberman, J. (2003). Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* **112**, 659-72.
40. Harrison, I. P. & Selemidis, S. (2014). Understanding the biology of reactive oxygen species and their link to cancer: NADPH oxidases as novel pharmacological targets. *Clin Exp Pharmacol Physiol* **41**, 533-42.
41. Piotrowska, A. & Bartnik, E. (2014). [The role of reactive oxygen species and mitochondria in aging]. *Postepy Biochem* **60**, 240-7.
42. Moazzen, H., Lu, X., Liu, M. & Feng, Q. (2015). Pregestational diabetes induces fetal coronary artery malformation via reactive oxygen species signaling. *Diabetes* **64**, 1431-43.

43. Thompson, K. H., Barta, C. A. & Orvig, C. (2006). Metal complexes of maltol and close analogues in medicinal inorganic chemistry. *Chem Soc Rev* **35**, 545-56.
44. Kostova, I. & Saso, L. (2013). Advances in research of Schiff-base metal complexes as potent antioxidants. *Curr Med Chem* **20**, 4609-32.
45. Yang, F. & Luo, J. (2015). Endoplasmic Reticulum Stress and Ethanol Neurotoxicity. *Biomolecules* **5**, 2538-53.
46. Bailey, S. M. & Cunningham, C. C. (1998). Acute and chronic ethanol increases reactive oxygen species generation and decreases viability in fresh, isolated rat hepatocytes. *Hepatology* **28**, 1318-26.
47. Suzuki, T., Matsuo, K., Sawaki, A., Mizuno, N., Hiraki, A., Kawase, T., Watanabe, M., Nakamura, T., Yamao, K., Tajima, K. & Tanaka, H. (2008). Alcohol drinking and one-carbon metabolism-related gene polymorphisms on pancreatic cancer risk. *Cancer Epidemiol Biomarkers Prev* **17**, 2742-7.
48. Adachi, M. & Ishii, H. (2002). Role of mitochondria in alcoholic liver injury. *Free Radic Biol Med* **32**, 487-91.
49. Saitz, R., Helmuth, E. D., Aromaa, S. E., Guard, A., Belanger, M. & Rosenbloom, D. L. (2004). Web-based screening and brief intervention for the spectrum of alcohol problems. *Prev Med* **39**, 969-75.
50. Bondy, S. C. (1992). Ethanol toxicity and oxidative stress. *Toxicol Lett* **63**, 231-41.
51. Enggasser, J. L., Hermos, J. A., Rubin, A., Lachowicz, M., Rybin, D., Brief, D. J., Roy, M., Helmuth, E., Rosenbloom, D. & Keane, T. M. (2015). Drinking goal choice and outcomes in a Web-based alcohol intervention: results from VetChange. *Addict Behav* **42**, 63-8.
52. Amagase, H., Petesch, B. L., Matsuura, H., Kasuga, S. & Itakura, Y. (2001). Intake of garlic and its bioactive components. *J Nutr* **131**, 955s-62s.
53. Moriguchi, T., Saito, H. & Nishiyama, N. (1996). Aged garlic extract prolongs longevity and improves spatial memory deficit in senescence-accelerated mouse. *Biol Pharm Bull* **19**, 305-7.
54. Nagae, S., Ushijima, M., Hatono, S., Imai, J., Kasuga, S., Matsuura, H., Itakura, Y. & Higashi, Y. (1994). Pharmacokinetics of the garlic compound S-allylcysteine. *Planta Med* **60**, 214-7.
55. Kodera, Y., Suzuki, A., Imada, O., Kasuga, S., Sumioka, I., Kanezawa, A., Taru, N., Fujikawa, M., Nagae, S., Masamoto, K., Maeshige, K. & Ono, K. (2002). Physical, chemical, and biological properties of s-allylcysteine, an amino acid derived from garlic. *J Agric Food Chem* **50**, 622-32.
56. Morihara, N., Hayama, M. & Fujii, H. (2011). Aged garlic extract scavenges superoxide radicals. *Plant Foods Hum Nutr* **66**, 17-21.

57. McLaughlin, F. & La Thangue, N. B. (2004). Histone deacetylase inhibitors open new doors in cancer therapy. *Biochem Pharmacol* **68**, 1139-44.
58. Woldemariam, G. A. & Mandal, S. S. (2008). Iron(III)-salen damages DNA and induces apoptosis in human cell via mitochondrial pathway. *J Inorg Biochem* **102**, 740-7.
59. Chandupatla, S. R. (2015). Antioxidant and anticancer properties of metallo-salens, ProQuest Dissertations Publishing.
60. Desoize, B. (2002). Cancer and metals and metal compounds: part II--cancer treatment. *Crit Rev Oncol Hematol* **42**, 213-5.
61. Shi, H., Jing, X., Wei, X., Perez, R. G., Ren, M., Zhang, X. & Lou, H. (2015). S-allyl cysteine activates the Nrf2-dependent antioxidant response and protects neurons against ischemic injury in vitro and in vivo. *J Neurochem* **133**, 298-308.
62. Besada, P., Mamedova, L., Thomas, C. J., Costanzi, S. & Jacobson, K. A. (2005). Design and synthesis of new bicyclic diketopiperazines as scaffolds for receptor probes of structurally diverse functionality. *Org Biomol Chem* **3**, 2016-25.
63. White, J. D., Xu, Q., Lee, C. S. & Valeriote, F. A. (2004). Total synthesis and biological evaluation of +-kalkitoxin, a cytotoxic metabolite of the cyanobacterium Lyngbya majuscula. *Org Biomol Chem* **2**, 2092-102.
64. Welch, C., Wuarin, L. & Sidell, N. (1992). Antiproliferative effect of the garlic compound S-allyl cysteine on human neuroblastoma cells in vitro. *Cancer Lett* **63**, 211-9.

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