Evaluating the dopaminergic neurotoxic effects of chemotherapeutics

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Abstract

Chemotherapy-induced neurotoxicity is one of the most common contraindication seen in the cancer survivors. Thus chemotherapy-induced neurotoxicity can adversely affect the clinical management of these patients. Cancer survivors during chemotherapy often complain about problems with memory retrieval, learning and concentration, which may persevere even post-treatment or never fully resolve. Role of chemotherapeutics in hippocampal neurotoxicity is well established and is referred to as chemobrain or chemofog. However, there are very few reports on the chemotherapeutics-induced neurotoxicity in movement disorders like Parkinson's disease. Hence, in this study we used dopaminergic cell lines (N27) to investigate the cytotoxic effects of doxorubicin and cyclophosphamide. N27 cells have been well established in studies of dopamine biosynthesis, neurotoxicity and used as a dopaminergic model for in vitro studies. In this study the dopaminergic neurotoxicity was evaluated in N27 cells using MTT assay. The effect of doxorubicin and cyclophosphamide were studied on markers of oxidative stress, mitochondrial functions and other relevant neurotoxic mechanisms. All statistical analyses were performed using the Prism-V software (La Jolla, CA, USA). All data were expressed as Mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by an appropriate post-hoc test including Tukey's and Dunnett's method ($p < 0.05$) was considered to indicate statistical significance). Doxorubicin significantly induced dopaminergic neurotoxicity. Thus, if careful patient care measures are not taken with cancer survivors exposed to doxorubicin and cyclophosphamide, it can considerably increase the risk for several movement disorders.

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List of Abbreviations

DOX Doxorubicin

CYPX Cyclophosphamide

GSK Glycogen synthase kinase

DA Dopamine

CRCI Chemotherapy related cognitive impairment

NF-κB Nuclear factor kappaB

Chapter one

1.0 Introduction:

Advances in cancer therapy with newer therapeutic agents and more effective treatment regime has significantly improved survival and reduced the risk of recurrence. In general, the cancer therapy constitutes of chemotherapy, radiotherapy and surgical approach. Conversely, as the cancer patients survive, the treatment-induces undesired adverse effects. The increase in survival rate post-chemotherapy is not without significant economical and negative health consequences. Chemotherapy is associated with cognitive impairment commonly refer to as "chemobrain" or "chemofog". The term chemobrain specifically is attributed to the memory impairment following cancer chemotherapy. The time line between onsets of memory impairment after chemotherapy is considerably variable. The duration of symptoms of chemobrain is still a subject of scientific discussion. Within 4 weeks of breast cancer chemotherapy, 20.0% and 6.7% of patients reported self-rated impairment of executive function and episodic memory, respectively (Kitahata et al., 2017). Up to 83% of breast cancer survivors who have received chemotherapy report some degree of cognitive dysfunction (Jenkins et al., 2006). However, there are evidences suggesting that symptoms may be transient (Wefel, Lenzi, Theriault, Davis, & Meyers, 2004). Symptoms of chemobrain ranges from difficulty undertaking and completing simple tasks including meal preparation, keeping track of and paying bills, or getting ready to go out, and as needing additional time to perform these tasks (Hodgson, Hutchinson, Wilson, & Nettelbeck, 2013). Furthermore, the impact of chemobrain on the quality of life cannot be over emphasized. It has been suggested that the cognitive deficits experienced by cancer patients with chemobrain result in difficulties in

maintaining relationships because of the compensatory mechanisms that patients adopt, such as distancing themselves in order to conceal the symptoms of chemobrain . The domains of cognition affected by chemotherapy include executive function, memory, verbal function, language, construction, concept formation, reasoning, perception, orientation and attention. It is difficult to grade the level of cognitive impairment related to different oncological condition and the causal agents among the different classes of chemotherapeutic agents.

Historically, chemotherapy related neurotoxicity has been known for the last few decades. Methylglyoxal bis-dihydrochloride has been used since 1955 for the treatment of acute myelocytic leukemia and the lymphomas. Methylglyoxal bis-dihydrochloride was found to cause severe sensory-motor neuropathy (Spaulding et al., 1982). In 1975, patients with metastatic gastrointestinal malignancy were treated with 5-fluorouracil (intravenously) and hydroxyurea (orally). This treatment resulted in unusually high incidence of dose-dependent neurotoxicity. The observed neurotoxicity was due to the biochemical interaction of 5- fluorouracil and hydroxyurea resulting in an inability to convert 5- fluorouracil to the active metabolite and the accumulation of its catabolic neurotoxic substances (Lokich, Pitman, & Skarin, 1975). Similarly, cancer patients treated with misonidazole developed dose-dependent neurotoxicity. Misonidazole induced peripheral neuropathy at low dose and convulsions with the higher dose (Saunders, Dische, Anderson, & Flockhart, 1978). Comparable to the above clinical incidences, patients with histologically proven advanced breast carcinoma and different tumors treated with vindesine also

exhibited severe neurotoxicity in addition to constipation, alopecia and flu-like syndrome (Smith & Powles, 1979). During a 5-year period from 1979 to 1983, patients in Denmark with metastatic non-seminomatous and extra gonadal germ cell cancer were treated with cisplatin, vinblastine, and bleomycin. This chemotherapy treatment regimen induced noticeable long-term neurotoxicity (Hansen, 1992). Cisplatin, vinblastine, and bleomycin induced axonal degeneration and parasympathetic nerve dysfunction which resulted in cancer patients experiencing peripheral sensory neuropathy and Raynaud's phenomenon. The mechanisms of neurotoxicity were attributed to the hyper-reactivity of the central sympathetic nervous system. Likewise, suramin treatment had to be discontinued in cancer patients because of dose-limiting toxicity consisting of a syndrome of malaise, fatigue, lethargy and axonal neuropathy (Eisenberger et al., 1993). More recently non-hodgkin's lymphoma cancer patients treated with rituximab and bendamustine also exhibited severe neurotoxicity (Jenkins et al., 2006). The neurotoxicity was attributed to reduced cellular energy (mitochondrial dysfunction) induced by inflammation and oxidative stress (Lacourt & Heijnen, 2017). Chemotherapy induces neurotoxicity that has been related to both central and peripheral nervous system. However, the cellular and molecular mechanism associated with different chemotherapy-induced neurotoxicity has not been well elucidated. Therefore, this review focuses on the toxic mechanisms associated with chemotherapy-induced neurotoxicity.

Figure-1: "Past to present": Evidence of chemotherapeutics-induced neurotoxicity:

1955: Methylglyoxal bis-dihydrochloride

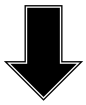
1975: 5- fluorouracil and hydroxyurea

1978: Misonidazole

1979: Vindesine

1992: Cisplatin, vinblastine, and bleomycin

1993: Suramin



2006: Rituximab and bendamustine

2. Chemotherapeutics:

In the bone marrow numerous types of cells are differentiated leading to cell growth. However, if this cell growth is uncontrolled it results in tumor or cancer. These uncontrolled cells growth can start altering the normal functioning of the cells and replace healthy cells. Various treatment has

been developed to treat cancer and it is selected depending on the type and stage of cancer. The various types of treatment are as follows:

- ✓ Surgery: Most effective treatment as of now to treat various cancers for many types of cancer today. In surgery, a surgeon will remove all the tumor and its surrounding tissue surgically. The surgeon does something called debulking (removing the cancerous tissue as much as possible which is done when an entire tumor cannot be removed. Sometimes reconstructive surgery is done which restores the body appearance and its functions. It can be done at the time of surgery too.
- ✓ Targeted Therapy: treatment which is very specifically targeted to cancerous cells to prevent them from spreading.

- ✓ Hormone Therapy: In certain breast cancers and prostate cancers this treatment has been proven effective where a patient is given hormones.

  Stem Cell Transplant: this is done to restore blood-forming stem cells in cancer patients who may have had destroyed while exposed to high doses of chemotherapy or radiation therapy.

- ✓ Radiation therapy: In this therapy, the oncologist uses high radiation to kill the cancerous cells. Radiation are these high energy waves which are known to induce to cause damage to the

intracellular organelles and preventing them from functioning well. which leading to cell death. Hence, radiation causes the tumor to shrink and the cancer cells to dies. Radiation interacts with nucleic acid and prevent them from undergoing mitosis ultimately leading to cell death. Radiation induced cell damage is manifested rapidly in tissues containing cells that are dividing rapidly. Radiation therapy is mainly used to treat breast, cervical and endometrium, choroidal melanoma, head and neck, Hodgkin's disease and prostate. Nevertheless, radiation therapy is not very effective in treating the following cancers like Wilms tumor, Rhabdomyosarcoma, colorectal cancer, soft tissue carcinoma, and embryonal carcinoma of testis. Commonly, there are two types of radiation therapy which are external beam radiation therapy and internal or brachytherapy. The issue is it is not specific to cancerous cells and alters the normal physiological cell functions. However, tumor cells do divide but this process is very slow and hence the size of the tumor is decreased. Radiation therapy will respond well if the treated carcinomas has the division rate very high. To prevent regrowth of the tumor radiation is often coupled with surgery and/or chemotherapy.

- ✓ Immunotherapy: In this approach, the drugs affect the immune system or function of the body. The mode of actions of these drugs is to stimulate the patient's innate immune system. The main types of immunotherapy are Monoclonal antibodies, Immune checkpoint inhibitors, Cancer vaccines, and non-specific immunotherapies.

- ✓ Chemotherapeutics are drugs that are indicated for cancer or tumors. These drugs prevent the cell division and inhibit the further growth of cells. Chemotherapy is a therapeutic approach of administrating a drug or a combination of drugs delivered in the form of topical ointment, a pill, or through an IV infusion. Various chemotherapeutics are alkylating agents, plant alkaloids, antimetabolites, antibiotics (antitumor), topoisomerase inhibitors and miscellaneous drugs.

2.1 a) Introduction of alkylating agents

Alkylating agents are the most traditional chemotherapeutic agents and are cell-cycle nonspecific chemotherapeutics. The stages of cell cycle are interphase (G_1, S, and G_2), M (mitotic), and cell cycle exit phase (Resting state, G_0, after the G_1 phase). Certain chemotherapeutics can inhibit the cell growth or kill the cells during any phase of the cell cycle and therefore referred to as cell-cycle nonspecific agents. Alkylating agents were the first chemical agents to demonstrate significant clinical regressions of tumors. The initial alkylating agents were nitrogen mustard compounds which were derived from sulfur mustard, a vesicant war gas (irritant chemical compounds which acted primarily by blistering the skin or mucous membranes). Sulfur mustard (mustard gas, military designation HD or H) was initially utilized in World War I. Sulfur mustard was found to be toxic to proliferating tissues and the less volatile nitrogen mustard compounds were found to have antitumor activity in animals and patients (Michael Colvin; 2002).

b) *Classification of alkylating agents:*

- **Nitrogen Mustards:** Nitrogen mustards are the most commonly used alkylating agents. Currently, only four nitrogen mustard (Cyclophosphamide, Melphalan, Chlorambucil and Ifosfamide) are used. Out of all the four nitrogen mustards, cyclophosphamide is the most widely used alkylating agent. These alkylating agents share the mechlorethamine group to exhibit their pharmacological action. The bischloroethyl group of nitrogen mustards form aziridinium intermediate which carry out its antitumor activity and the remaining part of the molecule determines its physicochemical, pharmacodynamics and pharmacokinetic properties. Cyclophosphamide is a pro-drug which gets activated by cytochrome P450 to produce 4-hydroxycyclophosphamide, which stays in equilibrium with aldophosphamide (Zon et al., 1984). Since 4-hydroxycyclophosphamide is relatively non-polar, it enters the target cells readily by diffusion. Aldophosphamide is spontaneously metabolized to produce phosphoramide mustard, which is cytotoxic, and carry out its antitumor activity (Fenselau et al., 1977).

- **Aziridines and Epoxides:** Aziridines are a class of chemotherapeutics that are very much similar to the nitrogen mustards. Currently used aziridines and epoxides are thiotepa, mitomycin C, and diaziquone (AZQ). Aziridines and epoxides act by the same mechanism as the aziridinium intermediates but the aziridine rings in these compounds are uncharged and are less reactive than the ones produced by nitrogen mustard.

- **Nitrosoureas:** This class of alkylating agents has been used since the past few decades (Hyde et al; 1962). Carmustine (BCNU) was the first nitrosourea tested for antineoplastic action. It is highly lipid soluble and readily crosses the blood brain barrier (BBB) and hence is currently

being used for the treatment of primary brain tumors, gliomas, melanoma, and lymphoma (Walker et al; 1978). These compounds metabolize to produce alkylating compounds which are cytotoxic under physiological conditions (Colvin, Brundrett, Cowens, Jardine, & Ludlum, 1976). They undergo metabolism to form chloroethyl diazonium moiety in basic conditions which then reacts with DNA to form a unique interstrand DNA cross-link (Kohn KW; 1977).

- **Triazenes & Hydrazines:** These classes of drugs are nitrogen-containing compounds that metabolize to produce alkyl diazonium intermediates that alkylate biological molecules. From this class of drugs, procarbazine and dacarbazine are metabolized to reactive intermediates that further decompose to form methyl diazonium, which then methylates DNA to produce methylazoxyprocarbazine (Erikson et al; 1989).

c) Mechanism of action of alkylating agents:

Alkylating agents act chemically with deoxyribonucleic acids (DNA) and proteins. Upon binding or interacting with the DNA, these alkylating agents react with guanine or cytosine bases, whereby substituting the alkyl radicals for hydrogen atoms. Alkylating agents react with electron-rich atoms to form covalent bonds. Alkylating agents can be mono-functional or bi-functional reacting with one strand of DNA or both strands of DNA respectively. This results in the breakage of DNA strand, DNA intrastand or interstrand cross links, or DNA–protein cross links. This prevents normal function of DNA (DNA replication and consequent RNA transcription and translation) at all phases of the cell cycle, which if left unrepaired, will prevent the cell from replicating and thereby inhibiting the growth or cell division (Rajesh Thirumaran and Paul B. Gilman; 2007).

Therefore these agents are cell cycle phase-nonspecific and are also fatal to resting cells. Thus, this group of chemotherapy drugs may be effective against slowly growing tumors as well as rapidly growing tumors (Jane M Dobson and Anne E Peaston; 2008).

d) Indication or Use of alkylating agents:

Ifosfamide is used in the treatment of sarcomas, lung cancer and testicular cancer. Cyclophosphamide is used in the treatment of lymphatic leukemia, Hodgkin's lymphoma and different disseminated solid tumors. Procarbazine is used in hematological malignancies and brain tumors (Carla et al; 2003). The carmustine (BCNU) and lomustine (CCNU) cross the blood-brain barrier easily. Carmustine and lomustine are used to treat primary brain tumors, glioma, melanoma and lymphoma (Walker et al; 1978). Due to the immunosuppressive effect, they are also used for the treatment of autoimmune disease where suppression of host's immunity is required.

e) Adverse Drug Reactions / Side effects of alkylating agents:

Alkylating agents cause alopecia and have immunosuppressive effects. They are also known to cause gastrointestinal side effects and dose-dependent toxicity to bone marrow. They produce an acute suppression of the bone marrow which also decreases the granulocyte count. Busulfan depresses all blood molecules, particularly stem cells. They also cause pulmonary fibrosis and hepatotoxicity. They are also toxic to mucosal cells causing oral mucosal ulceration and altering intestinal mucosa. Cyclophosphamide and ifosfamide cause a severe hemorrhagic cystitis due to their release of a toxic metabolite acrolein which can be treated by using thiol flushing agents. The

more unstable alkylating agents, such as nitrogen mustard and the nitrosoureas, have strong vesicant properties. Alkylating agents damage the blood vessels (veins) with repeated use and produce ulceration, if extravasated (Chabner et al; 2001, Pratt et al; 1994, Tew et al; 2000, Fraiser et al; 1991).

f) Hypersensitivity Reactions of alkylating agents*:*

The alkylating agents are known to react with many biological molecules and hence serve as haptens and produce allergic reactions (Cornwell et al; 1979). The most common immunogenic reactions of alkylating agents that have been reported are cutaneous hypersensitivity reactions. Patients treated with procarbazine developed macular-papular rashes or urticaria (Raymond; 1992). In another study, patients treated with Procarbazine for primary brain tumors experienced hypersensitivity reactions, cough, prominent eosinophilia and lung toxicity (Lehmann et al; 1997). The lung toxicity was similar to allergic alveolitis with interstitial pneumonitis (Lehmann et al; 1997). Cyclophosphamide and ifosfamide can cause dermatological hypersensitivity reactions and bullous rashes (Popescu et al; 1996). Chlorambucil also caused delayed rashes (toxic epidermal necrolysis). Melphalan (iv infusion) induced anaphylactic-like symptoms such as urticaria, angioedema, bronchospasm and hypotension (Kurer et al; 1995).

g) Effect on CNS by alkylating agents**:** Procarbazine can act as a monoamine oxidase inhibitor and produce acute hypertensive reactions after the ingestion of tyramine-rich foods (Auerbach; 1990). Oxazaphosphorines are formed as a result of metabolism of nitrogen mustard drugs like

cyclophosphamide and ifosfamide. Chloroacetaldehyde is a neurotoxic metabolite of Oxazaphosphorines. Ifosfamide and some of its metabolites cross the blood-brain barrier. About 45% of ifosfamide is usually metabolized to chloroacetaldehyde while in the case of cyclophosphamide only 10% of the parental drug is metabolized to chloroacetaldehyde. Therefore, cyclophosphamide has less neurotoxic effects as compared to ifosfamide (Kaijser et al; 1993). Ifosfamide (high doses) also causes acute, but usually reversible, encephalopathy. The encephalopathy is characterized by cerebellar symptoms, extrapyramidal symptoms, hallucinations, seizures and coma (DiMaggio et al; 1994). Symptoms usually start within 24 hours of the drug infusion and resolve in 3–4 days. In addition, a severe, painful axonal peripheral neuropathy is also associated with high-dose ifosfamide. These effects were seen in patients treated for bone and soft tissue sarcomas with ifosfamide (Patel et al; 1997). Patients receiving high-dose of cyclophosphamide has shown to induce reversible blurred vision, dizziness and confusion (Tashima CK; 1975). Nitrosoureas rarely cause neurotoxicity in conventional doses, however, patients with brain tumors who have received previous radiotherapy and are treated with high-dose or intra-arterial carmustine develop ocular toxicity, encephalopathy and seizures (Rosenblum et al; 1989). Sudden blindness due to optic neuropathy is a rare complication of oral lomustine therapy combined with cranial radiotherapy (Wilson et al; 1987).

h) Molecular mechanisms involved with the Cognitive impairment induced by alkylating agents:

Numerous clinical and animal studies have been focused on the effect of chemotherapeutics or post-treatment outcome on the cognitive function. Both standard-dose chemotherapy and high-

dose chemotherapy may cause cognitive impairment, consisting of problems with memory and concentration. Patients with breast cancer treated with high-dose cyclophosphamide, had an elevated risk for cognitive impairment 2 years after chemotherapy compared with a non-treated control group. In addition, patients treated with conventional cyclophosphamide showed a higher risk of cognitive impairment (Kirkwood et al; 1990). These results were also obtained in another study with long-term survivors of breast cancer and lymphoma, who were treated with standard-dose chemotherapy. Cognitive deficits were identified on average 10 years after chemotherapy. Therefore, persistent cognitive deficits may occur long after treatment (Auerbach; 1990). Hence, in this review, the effects of alkylating chemotherapeutics on various molecular signaling pathways associated with cognitive impairment were assessed.

Insulin-like growth factor 1 (IGF-1, IGF ligand) interacts with the growth hormone/IGF-1 axis by binding to the IGF-1 receptor. This binding results in the activation of the receptor (IGF-1R) through autophosphorylation and subsequent phosphorylation of insulin receptor substrate-1 (IRS-1). This can further trigger the Ras and Raf leading to ERK1/2 activation resulting in cell proliferation. However, alkylating chemotherapeutics significantly affect this molecular signaling by reducing / depleting IGF, decreasing the expression of Raf, Ras and ERK1/2 in the brain (Wadley et al., 2015)(Gaman, Uzoni, Popa-Wagner, Andrei, & Petcu, 2016b). The Ras genes play an important role in tumor development and represent one of the most common targets for gain-of-function mutations in cancer. The interaction of chemotherapeutics on the IGF pathway was further validated by IGF-1 administration to high-dose cyclophosphamide treated rodents

(Janelsins et al., 2010). IGF-1 significantly increased the neurogenesis (number of newly formed cells) in the dentate gyrus in mice that received chemotherapy(Janelsins et al., 2010). The PI3K–Akt pathway has a central role in cancer progression by enhancing metabolism, proliferation, survival and the transformation of cells. The phosphoinositide 3-kinases (PI3K) are activated by growth factors through receptor tyrosine kinases (RTKs), but can also be activated downstream of the Ras signaling cascade. In human cancers, PI3K signaling is activated through amplification of PI3K-α or Akt1, or through a loss of phosphatase and tensin homolog, a negative regulator of the pathway. Furthermore, PI3K activity can be increased through aberrant activation of upstream RTKs or the Ras cascade. Similarly, IGF-1 receptor stimulation can activate PI3K, which then leads to increased phosphatidylinositol 3,4,5-trisphosphate (PIP3), resulting in the phosphorylation (activation) of the AKT/PKB protein. This triggers AKT to release the anti-apoptotic protein Bcl-2 from Bad, activating protein synthesis through mTOR, and promoting glucose metabolism by inhibiting GSK-3β. This is commonly referred to as the PI3K/AKT pathway of IGF-1R signaling and is ultimately responsible for preventing cell death (Denduluri et al., 2015) Chemotherapeutics and/or its metabolites are known to decrease phosphorylation/activation of AKT signaling and decreases expression of GSK-3β, leading to cell death(Vallath, Hynds, Succony, Janes, & Giangreco, 2014)(Haldar et al., 2015). This alteration of the IGF molecular signaling by chemotherapeutics can decrease neurogenesis in hippocampal leading to neurodegeneration and cognitive impairment

Table-1 a:

Markers	Effect of Alkylating agents (CYP, BCNU, Busulfan)
IGF	Cyclophosphamide(CYP) increases the number of newly formed cells in the dentate gyrus in mice (Gaman, Uzoni, Popa-Wagner, Andrei, & Petcu, 2016a),(Y. C. Lee et al., 2010)
Raf	Carmustine (BCNU) generates ROS species that will activate ERK pathway to cause neuronal cell death and hence decreases raf signaling(Gaman et al., 2016b), (An et al., 2011) Busulfan treatment decreases raf signaling (Probin, Wang, Bai, & Zhou, 2006).
Ras	Carmustine generates ROS species that will activate ERK pathway to cause neuronal cell death, decreasing ras signaling (Gaman et al., 2016b)(An et al., 2011) Busulfan treatment also decreases ras signaling (Probin et al., 2006)

ERK1/2	Carmustine generates ROS species that will activate ERK pathway to cause neuronal cell death (An et al., 2011)(Gaman et al., 2016b) Busulfan treatment decreases the extracellular signal-regulated Erk activation (Probin et al., 2006)
Pi3K	Cyclophosphamide decreases the activation of Pi3K (Jeelani, Shaeib, Thakur, Khan, & Abu-Soud, 2016)(X.-Y. Chen, Xia, Guan, Li, & Zhang, 2016)
AKT	The metabolite of Cyclophosphamide, Acrolein, is known to decrease AKT signaling (Jeelani et al., 2016),(Haldar et al., 2015)) Carmustine treatment abolished AKT phosphorylation in castration resistant prostate cancer cells (CRPC) (Thamilselvan et al; 2016)
GSK-3β	Cyclophosphamide Inhibits expression of GSK-3β(Vallath et al., 2014) Carmustine also inhibits expression of GSK-3β (Thamilselvan et al; 2016)
mTOR	Cyclophosphamide decreases activation of mTOR (Jeelani et al., 2016)(X.-Y. Chen et al., 2016)

The sirtuins are a family of proteins that act predominantly as nicotinamide adenine dinucleotide (NAD)-dependent deacetylases. Sirtuins function as an anti-aging protein and the NAD requirement for sirtuin function indicates a link between aging and metabolism. SIRT1 controls mitochondrial function through the deacetylation of various targets that include PGC-1α and FOXO. The intracellular and particularly the nuclear levels of NAD are believed to increase activation of Sirt1 enzymatic activity. This leads to PGC-1α deacetylation, resulting in increased PGC-1α activity and an increase in mitochondrial number and function resulting in increased ATP production. Furthermore, SIRT1 is known to reduce the production of reactive oxygen species and stimulate oxidative energy production via the activation of AMPK, PPARα and PGC-1α, which simultaneously inhibit NF-κB signaling and suppress inflammation. The Hypoxia-Inducible

Factor (HIF) pathway mediates the primary cellular responses to low oxygen, which promotes both short- and long-term adaptation to hypoxia. Sirt1-mediated deacetylation of HIF-1α inhibits its transcriptional activity, whereas Sirt1-mediated deacetylation of HIF-2α potentiates its transcriptional activity. HIF rapidly increases oxygen supply through upregulation of the vasodilatory enzyme, inducible nitric oxide synthase. SIRT3 interacts with FOXO3 to activate antioxidant enzymes, manganese superoxide dismutase (MnSOD) and catalase to reduce the reactive oxygen species (ROS) (Sundaresan et al., 2009). The gene FOXO3 encoding the transcription factor forkhead box O-3 (FoxO3) is consistently associated with longevity in diverse human populations. SIRT3- knockout mice are prone to age- related disorders like cancer, cardiac hypertrophy, and metabolic syndrome. Excess generation of mitochondrial ROS is considered as a major cause of cellular oxidative stress. NADPH (intracellular reductant) has a key role in keeping glutathione in its reduced form GSH, which scavenges ROS and thus protects the cell from oxidative damage. SIRT desuccinylates and deglutarylates isocitrate dehydrogenase-2 (IDH2) and glucose- 6- phosphate dehydrogenase (G6PD) and thus activates both NADPH-producing enzymes. SIRT5 inactivation leads to high levels of cellular ROS and inhibition of IDH2 and G6PD, decreasing NADPH production, lowering GSH, impairing the ability to scavenge ROS and increasing cellular susceptibility to oxidative stress. An enhancement of sirtuin activity is associated with calorie restriction and life span increase in mammals. However, chemotherapeutics have shown to increase reactive oxygen species generation(An et al., 2011)(Gaman et al., 2016b), decrease the mitochondrial functions (Jeelani et al., 2016)(Sundaresan et al., 2009)affect NK-κB, HIF1-α, FOXO3 (Gaman et al., 2016b), decrease

TCA cycle enzymes (succinate dehydrogenase, malate dehydrogenase, and isocitrate dehydrogenase) which can lead to neurotoxicity and cognitive impairment

Table-1 b:

Markers	Effect of Alkylating agents (CYP, BCNU, Busulfan)
ROS	Carmustine, cyclophosphamide and busulfan increases ROS generation (Gaman et al., 2016b)(An et al., 2011).
Sirtuin	CYP significantly decreases the SIRT1 activity, while calorie restriction has shown to increase the SIRT1 activity (Xiang et al., 2012)
Mitochondrial function (PGC-1 Alpha Complex-I, IV activity, ATP content)	Cyclophosphamide decreases mitochondrial functions and ATP production (Probin et al., 2006)(Jeelani et al., 2016).
NK-κB	Cyclophosphamide is known to decrease production of NK-κB (Gaman et al., 2016b) Carmustine decreases production of NK-κB (Thamilselvan et al; 2017)
IDH2	A decrease in the activities of TCA cycle enzymes such as succinate dehydrogenase, malate dehydrogenase, and isocitrate dehydrogenase were noted in CYP-treated rats (Sundaresan et al., 2009)
FoxO3	Cyclophosphamide is known to affect FOX 03 (Obrador-Hevia, Serra-Sitjar, Rodríguez, Villalonga, & de Mattos, 2012)
HIF1-α	Cyclophosphamide is known to affect HIF1-α (Generali et al., 2015)

SOD2	Busulfan caused reduced levels of SOD-2 to be seen after treatment (Nasimi, Vahdati, Tabandeh, & Khatamsaz, 2016) Cyclophosphamide increases SOD activity ((El-Husseiny, Motawei, & Ali, 2016).

TNF receptor associated factor (TRAF) proteins mediate the signal transduction from members of the TNF receptor superfamily. This protein directly interacts with TNF receptors and forms a heterodimeric complex with TRAF1 which activates MAPK8/JNK. In a healthy cell, the outer membranes of its mitochondria display the protein Bcl-2 (anti-apoptotic) on their surface. Internal damage to the cell causes Bax to migrate to the surface of the mitochondrion, where it antagonizes the protective effect of Bcl-2 and inserts itself into the outer mitochondrial membrane, punching holes in it and causing cytochrome-C leakage. The released cytochrome-C binds to the protein apoptotic protease activating factor-1. Using the energy provided by ATP, these complexes aggregate to form apoptosomes, which in turn activate caspase-9. Caspase-9 cleaves and activates other caspases (caspase-3 and 7) and creates an expanding cascade of proteolytic activity which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and phagocytosis of the cell. Alkylating chemotherapeutics (Cyclophosphamide, Carmustine, Busulfan) has shown to affect Tumor necrosis factor (TNF)-receptor associated factor ((El-Husseiny et al., 2016),(Zhao & Kim, 2017). This triggers decreased expression of the antiapoptotic Bcl2 protein (Gibson, Ramirez, Maier, Castillo, & Das, 1999), (Nasimi et al., 2016)and increased expression of proapoptotic BAX protein (Gibson et al; 1999, Zhang et al; 2015, Garside et al; 2007, Qiao et al; 2016,(Nasimi et al., 2016)), which consequently cause pore formation in the mitochondria, leading to increases in cytochrome-C release (Qiao et al; 2016, Song et al; 2014),

activating caspases leading to neurodegeneration (Gaman et al., 2016b)(An et al., 2011)(Haldar et al., 2015),(Jeelani et al., 2016).

Table-1 c:

Markers	Effect of Alkylating agents (CYP, NCNU, Busulfan)
JNK	Carmustine increases generation of ROS species that activate c-jun N-terminal kinase (JNK) (Gaman et al., 2016b) Busulfan treatment induces c-Jun NH2-terminal kinase (JNK) activation (Probin et al., 2006)
BAD/BAX	Cyclophosphamide causes a dose and time dependent increase in BAX expression (Gibson et al., 1999) Carmustine increases BAX expression (Zhang et al; 2015, Garside et al; 2007). Busulfan increases Bax expression (Qiao et al; 2016,(Nasimi et al., 2016).
Bcl2	Cyclophosphamide showed reduced BCL-2 expression (Gibson et al; 1999). Carmustine showed reduced BcL2 expression (Lytle et al; 2004, Thamilselvan et al; 2016). Busulfan decreased expression of Bcl-2 (Qiao et al; 2016, (Nasimi et al., 2016)
Cytochrome-C	Busulfan increases cytochrome c release (Qiao et al; 2016, Song et al; 2014).
Caspases	Histological evaluation of carmustine treated rats shows increased expression of caspase-3 (An et al., 2011)(Gaman et al., 2016b) Busulfan induces caspase-3 activation (Toko et al; 2013, Ritam Chatterjee and Sujata Law; 2018). Cyclophosphamide is known to activate caspase-3 (Haldar et al., 2015)(Jeelani et al., 2016)

2.2 a) Introduction of plant alkaloids:

Plant alkaloids are chemotherapeutics and antitumor substances directly obtained or derived from plants. They are also commonly called "anti-microtubule" agents. Anti-microtubule agents inhibit cell division by preventing microtubule function. Microtubules consist of α-tubulin and β-tubulin proteins which are hollow, rod shaped structures that are important for cell division. Microtubules are structures that are permanently in a state of assembly and disassembly. Vinca alkaloids and taxanes are the two main groups of anti-microtubule agents that cause microtubule dysfunction via different mechanisms. The vinca alkaloids prevent the formation of the microtubules, while taxanes prevent the microtubule disassembly process which prevents completion of mitosis in cancer cells. Therefore, plant alkaloids cause cell cycle arrest which further induces programmed cell death (apoptosis). Correspondingly, these drugs also suppress blood vessel growth and hence prevent metastasis (Michael; 2013).

b) Classification of plant alkaloids*:* Plant alkaloids are classified into four groups:

- **Vinca alkaloids:** These drugs are obtained from the Madagascar periwinkle plant. The naturally occurring alkaloids are extracted from the pink periwinkle plant called *Catharanthus roseus* G. Don. Vinca alkaloids were traditionally used to treat diabetes mellitus because they exhibit an anti-hyperglycemic effect. The most commonly used from this class of drugs are vincristine (VCR), vinblastine and vinorelbine (VRL). Vinca alkaloids decrease the microtubule polymerization, which leads to microtubule depolymerization, and this causes inhibition of cell proliferation by arresting the cells at the metaphase stage of mitosis. Since

the microtubule is not able to form there will be no mitotic spindle formed, hence preventing chromosome segregation and blocking the cell division at G2/M phase (Mary et al; 1991).

- **Taxanes**: These are often called as antitubulins. Taxanes bind to dimeric tubulin, disrupting the microtubule network by inhibiting tubule disassembly, leading to stable microtubule bundles to accumulate in the cell. These kinds of cells are incapable of forming normal mitotic spindles, locking cells in the G2 and M phases of the cell cycle, unable to divide (Michael; 2013). Most commonly used taxanes are paclitaxel which is an active component of a bark extract from the Pacific yew *Taxus brevifolia*. Docetaxel is a semisynthetic product derived from the European yew *Taxus bacata*. Paclitaxel and docetaxel prevent the depolymerization of microtubules and prevent the progression of cells through the M-phase of the cell cycle.

- **Podophyllotoxins**: Podophyllotoxins are naturally occurring molecules derived from *Podophyllum peltatum* and *Podophyllum emodi*. Etoposide and teniposide are the commonly used podophyllotoxins. The mechanism of action of these drugs is to inhibit the cell growth by inhibiting DNA topoisomerase II. DNA transcription and replication results in significant tangling, leading to twist of DNA. DNA topoisomerases untangle twists in DNA and the DNA topoisomerases I and II present in cells act through scission of the DNA backbone on one or two strands, respectively. This is then followed by relief of torsional stress and then relegation of the broken DNA backbone. DNA topoisomerases are present in large complexes in the nucleus and control and carry out transcription and replication. Furthermore, they are also essential to maintaining chromatin organization and cell survival. Etoposide is relatively cell cycle specific, as

it affects cells in the S and G2 phases of cell division. However, it is considered highly toxic and many congeners have been made to minimize its toxicity.

- **Camptothecin analogs**: Camptothecin is a naturally occurring alkaloid which is derived from the plant called *Camptotheca acuminata*. Camptothecin is known to act by forming a stable ternary complex, which leads to inhibition of normal DNA relegation which ultimately causes the complex to collide with the replication fork, leading to a DNA double-strand break and cell death. Most commonly used in this class are Irinotecan and topotecan.

c) *Mechanisms of action of plant alkaloids:*

Disorganization of the microtubule structure can cause many effects like induction of tumor suppressor gene called p53 and causing activation or inactivation of protein kinases involved in cell signaling pathways like Ras/Raf, PKC/PKA (Wang et al, 1999a). These molecular changes result in phosphorylation and hence inactivation of the apoptosis inhibitor Bcl2 (Haldar et al, 1995). This in turn results in a decrease in the formation of hetero-dimers between Bcl2 and the pro-apoptotic gene, BAX, triggering the process of apoptosis in the cell (Wang et al, 1999). Inhibition of DNA topoisomerases by naturally occurring substances and synthetic drugs is an effective method for causing DNA damage due to the formation of irreversible covalent cross-links between the topoisomerase and DNA, stalling its replication (causing double strand breaks in DNA), and inducing cytotoxicity and apoptosis, thereby leading to cell death.

d) *Indication or Use of plant alkaloids:* Vinblastine is commonly used to treat Hodgkin's lymphoma (Arnold L. Demain and Preeti Vaishnav; 2011). VRL has been used for the initial treatment of patients with advanced lung cancer (Gregory RK, Smith IE; 2000). VCR has been approved to treat acute leukemia, rhabdomyosarcoma, neuroblastoma, Wilm's tumor, Hodgkin's disease and other lymphomas. Taxanes are commonly used to treat breast, prostate, and lung cancers (Jeffrey Schlom and James L. Gulley; 2013). Etoposide is used in the treatment of small cell lung carcinoma and testicular cancer. Irinotecan is approved by the Food and Drug Administration to treat colorectal cancer. Topotecan is used for ovarian, small cell and cervical cancer (Christopher D. Willey and James A. Bonner; 2012).

e) Adverse Drug Reactions / Side effects of plant alkaloids: The adverse reactions for Vinca alkaloids include neutropenia, thrombocytopenia and anemia. Reports of gastrointestinal toxicities like bloating, constipation, ileus and abdominal pain are found most commonly with vincristine. Nausea, vomiting, diarrhea, cardiac ischemia, chest pains, acute pulmonary effects have also been reported due to vinca alkaloids. These drugs are contraindicated in pregnancy for potentially causing birth defects (Kufe et al; 2003). Adverse drug reactions due to taxanes include urticaria, dermatitis and reactive erythema (Michael; 2009). The dose-limiting toxic reactions include myelosuppression, neutropenia, mild thrombocytopenia, hypotension, mucositis, nausea, alopecia, and emesis a (Bruce Montgomery and Daniel W. Lin; 2009).

f) *Hypersensitivity Reactions related to plant alkaloids:* Patients treated with taxanes showed higher incidence of hypersensitivity than the normal population of patients but the reaction to the

taxanes does not appear to be IgE related. Studies shows that these reactions are caused by drug-induced histamine release from basophils (Miles Hacker; 2009).

g) Effect on CNS by plant alkaloids: The neurotoxicity caused due to vinca alkaloids are peripheral and autonomic polyneuropathy. Pathology includes axonal degeneration and a decrease in axonal transport caused by a drug-induced perturbation of microtubule function. The uptake of VCR in the brain is low, but it still causes confusion, depression, agitation, insomnia, hallucinations, seizures, coma (Himes; 1991). The neuropathy is due to the adverse effect on small and large sensory fibers causing burning paresthesia of the hands and feet, leading to loss of reflexes. Motor and autonomic neuropathies have also been reported with both agents. Paclitaxel infrequently causes seizures and encephalopathy, but paclitaxel is administered with cremophor, leading to increased neurotoxicity (Arthur D. Forman and Victor A. Levin; 2009).

h) *Molecular mechanisms involved with the cognitive impairment induced by plant alkaloids*:
As previously described, plant alkaloids carry out their cytotoxic actions by altering functions of microtubules by either stabilizing or disrupting its assembly. Microtubules play a role in the development of neurons by providing structural support for neurons. Elongation of microtubules helps in the growth of neurites through interactions with the growth cone and takes part in mediating axonal transport in the neuron (Daniels, 1975) Since plant alkaloids are known to alter the microtubules, they can highly significantly impair this neuronal development. Abnormal microtubule arrays were seen in spinal cord-sensory ganglion explant cultures after exposure to paclitaxel resulting in abnormal neuronal growth and neuronal death (Lipton et al., 1989).

Interestingly, another study showed that paclitaxel accumulates in the dorsal root ganglia, sciatic nerve and spinal cord even at a very low dose (Tasnim et al., 2016). Presence of these drugs can induce neuropathy, leading to inappropriate growth of neuronal cells and ultimately, neuronal cell death.

Insulin and IGF-1 can stimulate the transcription of a Lef/Tcf-dependent luciferase reporter gene, which is mediated through the activation of phosphatidylinositol 3-kinase (PI3-K)/Akt and the inhibition of glycogen synthase kinase-3beta (GSK-3β). Paclitaxel has shown to decrease IGF signaling (Beech, Parekh, & Pang, n.d.). GSK3 beta plays an important role in the pathophysiology of several CNS and peripheral disorders such as non-insulin-dependent diabetes mellitus, cardiovascular disease, Alzheimer's disease, Parkinson's disease, Huntington's disease and bipolar disorder. Due to its role in the pathophysiology, GSK3beta inhibitors have significant therapeutic potential. GSK3beta also has an important function in the neoplastic transformation and tumor development (tumorigenesis and cancer progression). The role of GSK3beta is debatable because it can act as a "tumor suppressor" for some kinds of tumors, but also act as tumor growth promoter and help in development of certain other types of tumor/cancer. GSK3beta also mediates drug sensitivity/resistance in cancer chemotherapy. Rapamycin is known to activate GSK3β and enhances a paclitaxel-induced apoptosis in GSK3β wild-type, but not in GSK3β null breast cancer cells, indicating that GSK3β mediates rapamycin-induced chemosensitivity. In addition, GSK3β activation sensitizes human breast cancer cells to 5-fluorouracil, cisplatin, taxol or prodigiosin-induced apoptosis (J. Chen et al., 2009).

GSK-3 is a primary target of Akt, and inhibitory phosphorylation of GSK-3α (Ser21) or GSK-3β (Ser9) has various physiological actions. The above effects induce glycogen metabolism, cell cycle progression, regulation of wnt signaling and formation of neurofibrillary tangles in Alzheimer's disease. Insulin receptor signaling through Akt promotes Glut4 translocation through activation of AS160 and TBC1D1, resulting in increased glucose uptake. Akt regulates glycolysis through phosphorylation of PFK and hexokinase. Therefore, Akt is associated with glucose metabolism, insulin signaling, and promotes cell survival due to phosphorylation and inactivation of several pro-apoptotic agents such as Bad, Bim, Bax, and the forkhead (FoxO1/3a) transcription factors. Akt is one of the most frequently activated kinases in human cancer, as constitutively active Akt can promote unregulated cell proliferation. Plant alkaloid chemotherapeutics significantly affect this molecular signaling by reducing / depleting IGF, decreasing the expression of Raf, Ras, Pi3K and ERK1/2 ((Fan et al., 2000), (Tazi et al., 2017), (H. Shin, Jo, Kim, Kwon, & Myung, 2015), (Salerni, Bates, Albershardt, Lowrey, & Eastman, 2010), Liu et al; 2015). Disruption of IGF, mTOR and Akt signaling by plant alkaloids and can lead to cognitive impairment (Jones, Crombleholme, & Habli, 2014)

Table 2 a:

Markers	Effect of plant alkaloids (paclitaxel, vinblastine, etoposide)
IGF	The protein expression of IGF signaling molecules were reduced in the paclitaxel treated cancer cells (Qian et al; 2015).
Raf	Vinblastine decreases expression of raf (Fan et al; 2000)
Ras	Vinblastine decreases expression of ras (Fan et al; 2000)
ERK1/2	Vinblastine causes inactivation of ERK (Fan et al; 2000) Paclitaxel reduces expression of ERK (Ma et al; 2017). Etoposide inhibits ERK activation (Shin et al; 2016).
Pi3K	Etoposide inhibits PI3K activation (Lai et al; 2017, Yu et al; 2008). Vincristine shows synergistic effect with PI3K inhibitors in inhibiting the PI3K activation (Liu et al; 2015).
AKT	Paclitaxel treatment inhibited AKT signaling (Luo; 2009, Qian et al; 2015).
GSK-3β	Paclitaxel inhibits GSK-3β (Luo; 2009, Qian et al; 2015). Vinblastine inhibits GSK-3β (Naito et al; 2010).
mTOR	Vinblastine and Etoposide disrupts the mTOR signaling (Habli et al; 2017) Paclitaxel decreases mTOR signaling (Shafer et al; 2010).

Table 2 c:

Markers	Effect of plant alkaloids (vinblastine, paclitaxel, etoposide).
JNK	Vinblastine treatment induces c-Jun NH2-terminal kinase (JNK) activation. (Fan et al; 2000, Calviño et al; 2015). Paclitaxel treatment increases JNK activation (Jun-ichi Okano and Anil K. Rustgi; 2001).
BAD/BAX	Vinblastine treatment increases bax expression (Upreti et al; 2006).
Bcl2	Vinblastine increases phosphorylation of Bcl2 which leads to its inactivation (Haldar et al; 1995, Fan et al; 2000). Paclitaxel increases phosphorylation of BcL2 (Ma et al; 2017).
Cytochrome-C	Paclitaxel increases cytochrome c release (Jun-ichi Okano and Anil K. Rustgi; 2001). Vinblastine increases cytochrome c release (Upreti et al; 2006).
Caspases	Etoposide increases activation of caspases (Yu et al; 2017). Vinblastine increases activation of caspase-3 (Simizu et al; 1998).

Damage of the peripheral nerves and spinal cord by drugs and toxins can lead to peripheral neuropathy. Chemotherapy induced peripheral neuropathy (CIPN) is caused by neuronal

derangement due to chemotherapeutic agents. CIPN is a major dose limiting and disabling adverse drug reaction. Chemotherapeutics that induce CIPN are taxanes, Vinca alkaloids, platinum compounds and bortezomib. Vinca alkaloids induce CIPN by affecting nitric oxide and inflammatory mediators (Yu et al; 2007, Calviño et al; 2015, Jun-ichi Okano and Anil K. Rustgi; 2001, Aley K.O and Levine J.D; 2002, Press C and Milbrandt J; 2008). Vincristine can induce impairment of several genes associated with mitochondrial functions, leading to mitochondrial dysfunction and oxidative stress. Remarkably, the Vinca alkaloid induced axonopathy and axon transport disruption is the result of mitochondrial dysfunction and bioenergetic failure, and this is independent from the microtubule dysfunction (Bennett et al; 2014, Lobert et al; 1996). Plant alkaloid-induced toxicity occurs because of the generation of peroxynitrite which results in DNA damage. These plant alkaloids also activate poly ADP ribose polymerase (PARP) which directly activates the mitochondrial mediated apoptosis and causes neurodegeneration through bioenergetic failure (Brederson et al; 2012, Krantic et al; 2007). Furthermore, the Vinka alkaloids alter the transcriptional regulation of various nuclear genes (Smith et al; 2012, (Moudi, Go, Yien, & Nazre, 2013)Scarpulla; 2011, Austin S and St-Pierre J; 2012), Peroxisome proliferator activated receptor-γ coactivator-1 (PGC-1α). PGC-1α increases the synthesis of mitochondrial transcription factor A (Tfam), tri carboxylic acid cycle (TCA) and fatty acid oxidation through nuclear respiratory factor-1 (NRF-1) expression. PGC-1α upregulation enhances the nuclear erythroid factor (NEF)-2 related factor (Nrf-2) and thereby facilitating the production of glutathione (GSH), superoxide dismutase (SOD), glutathione-s-transferase (GST), Hemeoxygenase-1 (HO-1), NADPH Quinone oxidase reductase-1 (NQOR-1). Several drugs are known to act as PGC-1α

activators and proven to have therapeutic potential in many neurodegenerative disorders (Areti et al; 2016). However, Vinca alkaloids have shown to significantly affect this pathway, depleting ATP, inducing oxidative stress and decreasing the mitochondrial functions (Areti et al; 2016, Upreti et al; 2006, Shin et al; 2016). Hypoxia-inducible factor-1 (HIF-1) regulates several genes associated with cellular adaptation and cell survival under hypoxia. HIF-1 activation is involved in tumorigenesis, vascular remodeling, inflammation, and hypoxia/ischemia-related tissue damage. HIF-1 activation is associated with the pathophysiology of cancer and cancer treatment. Currently, HIF-1 inhibition is considered as a novel molecular target cancer therapy. Substances obtained /derived from botanicals, animals and microorganisms have been tested for their HIF-inhibition property. Microtubule disrupting agents like Vinca has been shown to inhibit the HIF pathway (Nagle and Zhou; 2006)

(Table 2b)

Markers	Effect of plant alkalaoids (vinblastine, paclitaxel, etoposide)
ROS	Etoposide increases generation of ROS species (Shin et al; 2016).
PGC-1 Alpha	Vinblastine decreases the expression of PGC-1 Alpha (Areti et al; 2016).
NK-κB	Etoposide is known to decrease production of NK-κB (Yu et al; 2007, Calviño et al; 2015). Paclitaxel decreases production of NK-κB (Jun-ichi Okano and Anil K. Rustgi; 2001).
HIF-alpha	Vinblastine inhibits HIF-alpha (Nagle and Zhou; 2006).

SOD2	Vinblastine decreases SOD2 activity (Areti et al; 2016).
Complex-I, IV	Vinblastine decreases Complex I-IV activity (Areti et al; 2016).
ATP	Vinblastine depletes ATP (Upreti et al; 2006).

There are several reports that shows that plant alkaloids induce apoptosis. Vinca alkaloid has shown to induce apoptosis without affecting G (2)-M arrest (Huang et al; 2004). Vinca alkaloids cause significant degradation of IκBα, which in turn results in nuclear factor-κB (NF-κB) activation. Plant alkaloids have shown to increase JNK activation (Fan et al; 2000, Calviño et al; 2015, Jun-ichi Okano and Anil K. Rustgi; 2001), increase pro-apoptotic factors expression (Upreti et al; 2006), decrease anti-apoptotic factors expression (Haldar et al; 1995, Fan et al; 2000, Ma et al; 2017), leading to caspase activation (Jun-ichi Okano and Anil K. Rustgi; 2001, Upreti et al; 2006, Yu et al; 2017, Simizu et al; 1998) and cell death. The Inhibitor of Apoptosis (IAP) proteins act downstream for a broad range of stimuli (cytokines and extracellular matrix interactions) to regulate cell survival, proliferation, and migration. IAPs are commonly upregulated in cancer. The survivin gene is among the top 5 cancer-associated genes. It is upregulated in the vast majority of cancers and is associated with resistance to both chemo and radio-therapy, as well as a poor prognosis. Survivin act to stabilize microtubules and this result in the resistance to chemotherapeutics.

2.3 a) Introduction of Antimetabolites:

Antimetabolite chemotherapeutics were one of the first drugs used to treat cancer. Antimetabolite chemotherapeutics are low molecular weight drugs and have structural resemblance to endogenous substances that are utilized by the body to synthesize nucleic acid (DNA and RNA). In general, Antimetabolite chemotherapeutics competitively inhibit the binding of the synthesizing enzymes (to the substrate and they also incorporate into the host nucleic acids, constraining the normal physiological effect (make them incapable of dividing / replicating) and triggering the apoptosis. Unlike other chemotherapeutics, Antimetabolites are cell-cycle specific drugs.

(Carmen Avendaño and J. Carlos Menéndez; 2008).

b) Classification of antimetabolites*:*

Antimetabolite chemotherapeutics are classified based on their action on the specific target components such as folic acid, pyrimidine and purine. Antimetabolite chemotherapeutics block the cell division by antagonizing the effects of folic acid, pyrimidine and purine.

- **Folic acid antagonist**: With regard to chemotherapy, Methotrexate is the most widely used folic acid antagonist (Chris; 1996). As the name suggests these classes of drugs interfere in production of folic acid. Methotrexate blocks the production of folate by inhibiting dihydrofolate reductase (DHFR). Folates are required for de novo synthesis of purines which

are required for DNA. They also exert their pharmacological action by attacking tumor cell division during the S phase, causing cell cycle arrest (Rajesh et al; 2007).

- **Pyrimidine antagonist:** Pyrimidine antagonists have structural similarity with naturally occurring nucleotides and they inhibit the synthesis of nucleic acids (Maring et al; 2005). The 5-fluorouracil (5-FU) is an uracil nucleotide and is the most prevalently used chemotherapeutic in this class of Antimetabolites. In the body, 5-FU is metabolized to fluoro-dUMP. Fluoro-dUMP incorporates itself into the nucleic acid and affects the RNA processing, DNA transcription and synthesis (Rajesh et al; 2007).
- **Purine antagonist**: The 6-mercaptopurine is the most common used chemotherapeutic in this class of Antimetabolites. 6-mercaptopurine is a purine analog and is also a cell cycle-specific chemotherapeutic. Regarding the cell cycle, 6-mercaptopurine acts in the S phase. 6-mercaptopurine is phosphorylated intracellularly by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and is converted to a cytotoxic monophosphate derivative. This toxic derivative inhibits the de novo purine synthesis by inhibiting the 5-phosphoribosyl-1-pyrophosphate amidotransferase (Rajesh et al; 2007).

c) Mechanisms of action of Antimetabolite Chemotherapeutics: Generally, antimetabolites act by mimicking purines and pyrimidines that are required for DNA synthesis. They most commonly affect cells in the S phase of the cell cycle, which is the phase of DNA replication (Zoltan Szucs and Robin L. Jones; 2016). Antimetabolites usually are known to disrupt nucleic acid synthesis by interfering with the production of the nucleotide metabolite or by substituting themselves in the

place of natural metabolite. Like most cytotoxic anticancer agents, antimetabolites are toxic to normal cells (Eric et al; 2007).

d) Indication or Use of antimetabolites: Methotrexate is used to treat acute leukemia, non-Hodgkin's lymphoma, colon, breast cancer, head & neck cancer, choriocarcinoma, osteogenic sarcoma and bladder cancer (Chris; 1996). 6-Mercaptopurine is used in the treatment of acute lymphocytic leukemia (Rajesh et al; 2007). 5-FU is mainly used in the treatment of colorectal, breast and head and neck cancer (Maring et al; 2005).

e) Adverse Drug Reactions / Side effects of antimetabolites: Adverse reactions related to 6-Mercaptopurine include myelosuppression, mucositis, diarrhea, abnormal liver function, nausea/vomiting, urticaria, and teratogenicity. Adverse reactions of 5-Fluorouracil include myelosuppression, mucositis, diarrhea, hand-foot syndrome, cerebellar ataxia, chest pain, and blepharitis. Adverse of reactions of methotrexate include myelosuppression, renal failure, cerebral dysfunction, pneumonitis, skin rash, abnormal liver function, mucositis and fetal death (Rajesh et al; 2007).

f) Hypersensitivity Reactions related to antimetabolites: There were some reports of anaphylaxis, urticaria and hepatitis after the exposure to methotrexate (Alkins et al; 1996). 5-FU is usually known to have less hypersensitivity reactions as compared to other drugs. However, there are

some very rare cases of anaphylactic reactions taking place during or after the intravenous injections (BISWAL, 1999).

g) Effect of antimetabolites on CNS:

Methotrexate is known to cause aseptic meningitis, subacute neurological toxicities consisting of hemiparesis, confusion, ataxia and seizures (Bates et al; 1985). Moreover, methotrexate has shown to induce progressive dementia, gait disturbances, hemiparesis, aphasia and death (Blay et al., 1998). In general, 5-FU doesn't have significant effect on the CNS but induce encephalopathy and coma in patients with dihydropyrimidine dehydrogenase deficiency; dihydropyrimidine dehydrogenase is an enzyme responsible for the rate-limiting metabolic clearance of 5-FU (Takimoto et al; 1996). Rarely occurring adverse effects of 5-FU treatment are cerebellar ataxia, extraocular muscle abnormalities, optic nerve neuropathy and extra-pyramidal syndromes (Bixenman et al; 1977).

h) Molecular mechanisms involved with the cognitive impairment induced by antimetabolites: Recent studies regarding chemotherapy have been focused on normal neurological function in patients receiving chemotherapy. It is said that both the standard dose and high dose of chemotherapy can lead to cognitive impairment that can lead to problems with memory. Reports show that standard doses of methotrexate and 5-FU have higher risk of cognitive impairment (Van et al; 1998). Cognitive impairment is known to occur on an average of 10 years after chemotherapy (Carla et al; 2003). The expression of the insulin-like growth factor receptor

is significantly increased on various cancer cells, and this leads to metastasis and resistance to apoptosis. Therefore, IGF-chemotherapy conjugates can considerably escalate the drug specific localization and express more beneficial antitumor effect (McTavish, Griffin, Terai, & Dudek, 2009). IGF-methotrexate conjugate was more effective than free methotrexate to inhibit *in vivo* tumor growth (McTavish et al., 2009). Following the Antimetabolite (5-fluorouracil and 5-fluorodeoxyuridine (floxuridine), a 5-fluorouracil metabolite) chemotherapy, Uracil N-glycosylase 2 (UNG2) catalyzes the removal of uracil or 5-fluorouracil from the nucleic acid (specifically DNA). Therefore, UNG2 promotes cancer cell survival and is therefore critical in tumor resistance. Interestingly, GSK-3 phosphorylation facilitates UNG2-dependent repair of floxuridine-induced DNA lesions and promotes tumor cell survival (Baehr et al., 2016). Antimetabolite chemotherapeutics have also shown to affect IGF signaling by affecting the Ras, Raf, ERK1/2, Pi3K, AKT and mTOR (Teachey, Grupp, & Brown, 2009).

Thymidylate synthase catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) by N (5), N (10)-methyhlenetetrahydrofolate, forming dTMP for the maintenance of DNA replication and repair. Inhibitors of TYMS have been widely used in the treatment of neoplastic disease. However, TYMS inhibitors induce oxidative stress that leads to cell death (Ozer et al; 2016). Methotrexate is one of the oldest chemotherapeutics. Though the inhibition of dihydrofolate reductase by methotrexate is the accepted pharmacological effect, its non-dihydrofolate reductase dependent mechanisms alter other metabolic pathways resulting in a depletion of NAD(P)H and an induction of oxidative stress by generating ROS (Hess and Khasawneh; 2015, Maruf et al; 2018,(Focaccetti et al., 2015). Methotrexate induces oxidative

tissue by increasing lipid peroxidation and decreasing the activity of glutathione peroxidase and superoxide dismutase ("Oxidative effect of methotrexate administration in spinal cord of rabbits," n.d.),(Barbisan et al., 2014). During hypoxia under a hypovascular environment, the transcription factor hypoxia-inducible factor-1α (HIF-1α) is activated. Hypoxia potentiates leukemic cell resistance to methotrexate directly by induction of expression of multiple anti-apoptotic proteins Bcl-2 (Lou et al., 2010)

Table 3 a:

Markers	Effect of antimetabolites (5-fluorouracil, 6-Mercaptopurine, methotrexate)
IGF	Methotrexate and IGF conjugate inhibits the IGF pathway and carry out cell growth inhibition (Mctavish et al; 2009).
Raf	5-Furouracil decreases expression of raf (Tseng et al; 2003).
Ras	5-Furouracil decreases expression of ras (Tseng et al; 2003).

ERK1/2	5-Furouracil causes inactivation of ERK (Tseng et al; 2003).
Pi3K	5-Furouracil inhibits PI3K activation (Tseng et al; 2003). 6-Mercaptopurine inhibits PI3K activation (Huang et al; 2016).
AKT	Methotrexate treatment inhibited AKT signaling (Cho et al; 2013) 6-Mercaptopurine treatment inhibited AKT signaling (Huang et al; 2016).
GSK-3β	5-Fluorouracil decreases GSK-3β expression (Baehr et al; 2016).
mTOR	Methotrexate treatment synergistically inhibits mTOR signaling along with mTOR inhibitors (Teachey et al; 2008). 6-Mercaptopurine treatment inhibits mTOR signaling (Huang et al; 2016).

Moreover, mitochondria have an important role in preserving the viability of the cancer cell viability and alterations in metabolism seen in cancer cells aid this mitochondrial ability. As controllers of energy need and nutritional energy providers, the mitochondria play an important role in cell physiology. PGC-1 family of transcriptional coactivators balances the cell growth with mitochondrial energy production (Cho et al; 2010). In addition to the well-known above physiological function, PGC-1 coactivators also play an important role in carcinoma. PGC-1α activity is elevated in breast cancers and are dependent on a resistant folate cycle and thereby more vulnerable to methotrexate. In the mitochondria, Methotrexate caused swelling and depleted glutathione and ATP (Maruf et al., 2017).

Table 3 b:

Markers	Effect of antimetabolites (5-fluorouracil, 6-Mercaptopurine, methotrexate)
ROS	Methotrexate increases production of ROS species (Maruf et al; 2018, Joshua A. Hess Mohamad K.Khasawnehb; 2015). 5-Fluorouracil increases production of ROS species (Focaccetti et al, 2015). 6-Mercaptopurine increases production of ROS species (Rehman et al; 2015, Huang et al; 2016, Morgan et al; 2015).
PGC-1 Alpha	5-Fluorouracil treatment decreases PGC-1 alpha (Sunanda; 2014).
NK-κB	Methotrexate suppresses NK-κB expression (Sekhar Majumdar and Bharat B. Aggarwal; 2001). 6-Mercaptopurine suppresses NK-κB expression (Huang et al; 2016, Kurakula et al; 2015).
HIF-alpha	Methotrexate activates HIF-alpha (Petit et al; 2016).
SOD2	Methotrexate treatment decreases SOD2 activity (Barbisan et al; 2014).
ATP	Methotrexate decreases ATP production (Maruf et al; 2018, Maruf et al; 2017). 5-Fluorouracil decreases ATP production (Sunanda; 2014).

TNF-induced activation of the transcription factor NF-kB and the c-jun N-terminal kinase (JNKySAPK) requires TNF receptor-associated factor 2 (TRAF2). The NF-kB inducing kinase (NIK) associates with TRAF2 and mediates TNF activation of NF-kB Tumor Necrosis Factor Receptor 2 (TNFR2) activates transcription factor κB (NF-κB) and c-Jun N-terminal kinase (JNK). The physiological effects triggered by TNFR2 depend on the recruitment of TNF Receptor-

Associated Factor 2 (TRAF2) to the intracellular region of the receptor. Jun N-terminal kinases (JNKs) belong to the superfamily of MAP-kinases. They control cell differentiation & proliferation and programmed cell death. JNKs-mediated signaling triggers death receptor-initiated extrinsic as well as mitochondrial intrinsic apoptotic pathways. This cell signaling pathway triggers apoptosis by upregulation of pro-apoptotic genes via the transactivation of specific transcription factors or by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through distinct phosphorylation events (Dhanasekaran and Reddy; 2008). Antimetabolite chemotherapeutics has shown to significantly affect the JNK signaling (Cho et al; 2013, Huang et al; 2016) and increase the risk for apoptosis. Antimetabolite chemotherapeutics have shown to increases BAX expression (Cho et al; 2013, Yang et al; 2009, Morgan et al; 2015), decrease Bcl2 expression (Cho et al; 2013, Tseng et al; 2003), increases cytochrome C release Yang et al; 2009,(Maruf et al., 2017) and increase caspases activation leading to cell death (Morgan, Parsels, Maybaum, & Lawrence, 2014).

Table 3 c:

Markers	Effect of antimetabolites (6- mercaptopurine, 5-fluorouracil, methotrexate).
JNK	Methotrexate treatment induces c-Jun NH2-terminal kinase (JNK) activation (Cho et al; 2013). 6-Mercaptopurine treatment induces JNK activation (Huang et al; 2016).
BAD/BAX	Methotrexate treatment increases BAX expression (Cho et al; 2013). 5-Furouracil treatment increases BAX expression (Yang et al; 2009). 6-Mercaptopurine increases BAX expression (Morgan et al; 2015)

Bcl2	Methotrexate decreases Bcl2 expression (Cho et al; 2013). 5-Furouracil decreases Bcl2 expression (Tseng et al; 2003). 6-Mercaptopurine decreases BCL2 expression (Morgan et al; 2015)
Cytochrome-C	Methotrexate increases cytochrome C release (Maruf et al; 2018). 5-Furouracil increases cytochrome C release (Yang et al; 2009). 6-Mercaptopurine increases cytochrome C release (Morgan et al; 2015)
Caspases	Methotrexate increases activation of caspase-9 (Nihal et al; 2014, Cho et al; 2013). 5-Furouracil increases activation of caspase-3 (Wu et al; 2001, Yang et al; 2009). 6-Mercaptopurine increases activation of caspase-3 (Morgan et al; 2015)

2.4 Introduction to antitumor antibiotics:

Antitumor antibiotic chemotherapeutics target the nucleic acid (mainly affect the function of DNA) to exert its action in the body. Interestingly, antitumor antibiotics differ from other antibiotics that are generally used to treat infections. However, due to their effects on DNA, these antibiotic chemotherapeutics retard or inhibit the growth of cancer cells. Usually, these chemotherapeutics are obtained from the natural products, specifically from fungi (Streptomyces species found in the soil). Antitumor antibiotics act at multiple phases of the cell cycle and are considered to be cell-cycle specific chemotherapeutics.

a) Classification of antitumor antibiotics:

i. Anthracyclines: The anthracyclines include daunorubicin, doxorubicin, epirubicin, and idarubicin. The antitumor antibiotic drugs daunorubicin and adriamycin (doxorubicin) were

initially isolated from *Streptomyces peucetius varcaesitue* (Behal; 2000) and exhibited the widest spectrum of antitumor activity against human cancers. Broadly, these chemotherapeutics also belong to the family of antibiotics. Pharmacologically, the anthracyclines prevent cell division by acting on DNA, where they disrupt the anatomy of the DNA and affect its physiological function. Regarding the specific mechanism of action, anthracyclines affect the DNA by interacting and intercalating into the base pairs in the minor grooves. Additionally, anthracyclines also induce the production of free radicals and damage the ribose in the DNA. Doxorubicin is the lead compound of the anthracycline family. Doxorubicin is a cell cycle-nonspecific drug but is most active in the S phase.

ii. Chromomycins: Actinomycin was first isolated from *Streptomyces antibioticus* and has been widely used in clinical practice since 1954 as an anticancer drug to treat tumors. Dactinomycin is a well-known antibiotic of this class of antitumor antibiotic chemotherapeutics. It is well known for its high antibacterial and antitumor activity. The mechanisms of action for its antitumor action is through the disruption of the DNA functionality by binding to single- and double-stranded DNA, which then affects the RNA, leading to the inhibition of protein synthesis (Koba M and Konopa; 2005). This action is primarily cell cycle nonspecific, although the effect peaks in the G1 phase.

iii. Other Antitumor drugs: Bleomycins (BLMs) are a family of natural glycopeptidic antibiotics produced by *Streptomyces verticillus* with clinical efficacy against several types of tumors such as squamous cell carcinoma, testicular carcinoma, and malignant lymphomas. Bleomycin inhibits Thymidine nucleotide incorporation during DNA synthesis and induces breakage of the DNA strands. Bleomycin inflicts direct damage to the DNA by generating chromosomal gaps, deletions

and DNA fragmentation. The activity of Bleomycin is largely dependent on the presence of oxygen and metal ions (primarily copper and iron), with which it chelates, forming a pseudoenzyme. The pseudoenzyme then reacts with oxygen to form highly reactive free radicals, which then bind to G-C rich regions of DNA and cleaves it. The cytotoxicity of Bleomycin is specific for the G2 phase of the cell cycle. The chelations also trigger peroxidation of cellular lipids and induce oxidation of other molecules within the cell (Sankaranarayanan et al; 2014).

b) General Mechanisms of action of antibiotics regarding Cancer/tumor: Antitumor antibiotics act by blocking the synthesis of DNA. They also inhibit RNA synthesis by binding to guanine residues and inhibiting DNA-dependent RNA polymerase (Kleeff, Kornmann, Sawhney, & Korc, 2000). The two main mechanisms are the intercalation of DNA and the stabilization of cleavable complexes of topoisomerases I and II with DNA. In the stabilization process, a phenoxazone ring localizes between the base pair sequence in DNA and polypeptide lactones rings occupy a position in the minor groove of the DNA helix or the drug binds to a place in the DNA structure where topoisomerase binds with DNA (Koba M and Konopa; 2005). The physical structure of doxorubicin allows it to intercalate into the DNA double helix, but the anticancer activity of the drug is believed to result from the induction of free radical formation and topoisomerase II-dependent DNA cleavage (Jane et al; 2008).

c) Indication or Use of antibiotic chemotherapeutics: Doxorubicin is used in the treatment of several cancers including breast, lung, gastric, ovarian, thyroid, non-Hodgkin's and Hodgkin's

lymphoma, multiple myeloma, sarcoma, and pediatric cancers (Caroline et al; 2011). Dactinomycin is used to treat sarcomas, pediatric solid tumors (Wilms' tumor, a type of renal tumor), germ cell cancers (testicular cancer) and choriocarcinoma (Carmen Avendaño and Carlos Menéndez; 2008). These chemotherapeutics are also used in the treatment of squamous cell cancers, melanoma, sarcoma, testicular cancer and both Hodgkin's and non-Hodgkin's lymphoma (Sankaranarayanan, Ramadas, & Qiao, 2014).

d) Adverse Drug Reactions / Side effects of antibiotic chemotherapeutics: Anthracyclines like doxorubicin can negatively affect noncancerous tissues, including striated muscle, contributing to the fatigue and muscle weakness in patients treated with anthracycline-based chemotherapy (Laura A.A. Gilliam and Daret K. St. Clair; 2011). The main dose-limiting side effect of this anthracycline class of compounds is cardiotoxicity, leading to heart failure in the most severe cases (Guido; 2007). The side effects of Bleomycin include skin rash, hives, changes in skin sensation, hyper-pigmentation (darkening of skin and nails), hair loss, mouth ulcers and hard patches on skin (Sankaranarayanan et al; 2014).

e) Hypersensitivity Reactions due to antibiotics: With Doxorubicin there are reports of Urticarial rash with pruritus (Solimando DA Jr and Wilson JP; 1984). Approximately 1% of lymphoma patients treated with bleomycin developed hypotension, mental confusion, fever, chills, and wheezing after the first or second dose (Gillian; 2003).

f) Effect of antibiotics on CNS: Adriamycin induces a severe form of myelopathy and encephalopathy. It also causes cerebral damage after intracarotid injection and anterior horn cell loss after direct injection into a peripheral nerve. Actinomycin D causes tremors, myoclonus, seizure, ascending myelopathy, and encephalopathy when it is injected into the CSF in animal models (Katie Kompoliti and Stacy S. Horn; 2007).

g) Molecular mechanisms involved with the cognitive impairment induced by antibiotic chemotherapeutics: Insulin-like growth factor (IGF) signaling prevents apoptosis and stimulates cellular differentiation and cellular proliferation, regulating growth and development of tissues in human. The IGF axis contains insulin and two related ligands such as IGF ligands 1 and 2 (IGF-1 and IGF-2). These ligands interact with the cell-surface receptors and regulate cellular processes. The insulin receptor, IGF-1R is a member of the receptor tyrosine kinase (RTK) class 2 family of receptors (insulin receptor family) and binds the IGF ligand. IGFs bind with higher affinity to the receptors when compared with insulin. IGF-2 binds to INSR-A, a fetal isoform that is overexpressed in some tumors, and to IGF-2 receptor (IGF-2R), a structurally unrelated receptor that lacks tyrosine kinase. Recruitment of IRS activates signaling via the PI3K/Akt and Ras/Raf/MAPK pathways, which regulate cellular proliferation, survival, migration, and metabolism. Furthermore, interactions between IGF-1R and integrins regulate cellular adhesion and motility via scaffolding with RACK1 and FAK proteins to these signaling pathways. Antitumor antibiotics have shown to significantly affect the IGF pathway by decreasing the expression of IGF, Raf, Ras (Fabbi et al., 2015), (Hughes & Andersson, 2017). Additionally,

antitumor antibiotics affect ERK1/2, Pi3K, AKT, GSK-3β, mTOR expression lead to significant neurotoxicity.

Table 4 a:

Markers	Effect of antibiotics (Doxorubicin, Actinomycin D, Bleomycin)
IGF	Doxorubicin decreases expression of IGF (Fabbi et al; 2015). Dactinomycin increases at first after 4 hours of addition and then declines (Johnson et as; 1991).
Raf	Actinomycin D decreases Raf activation (Hughes & Andersson, 2017)
Ras	Actinomycin D decreases Ras activation (Hughes & Andersson, 2017)
ERK1/2	Doxorubicin causes inactivation of ERK (Zhimin Lu and Shuichan Xu; 2006). Bleomycin treatment decreases ERK activation (Yang et al; 2004). Actinomycin D decreases ERK activation (Hughes et al; 1997).
Pi3K	Doxorubicin synergistically inhibits PI3K activation with kinase inhibitors (Babichev et al; 2016).
AKT	Doxorubicin treatment inhibited AKT signaling (Babichev et al; 2016). Actinomycin D treatment increased phosphorylation of AKT (Strohm et al; 2002).
GSK-3β	Doxorubicin decreases GSK 3beta expression (NGOK-NGAM et al; 2013).
mTOR	Doxorubicin treatment synergistically inhibits mTOR signaling along with mTOR inhibitors (Babichev et al; 2016).

PGC-1 Alpha (Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1-Alpha) is a tissue-specific transcriptional coactivator. PGC-1 Alpha is mainly expressed in mitochondrial-rich

tissues with high energy demands such as heart, brown adipose, skeletal muscle, liver and brain tissue. PGC-1 Alpha augments the activity of several nuclear receptors and organizes transcriptional programs imperative for cellular energy metabolism and energy homeostasis. PGC-1 Alpha is a key regulator of mitochondrial functional capacity and participates in the transduction of physiological stimuli to energy production. The expression of the PGC1 Alpha gene is upregulated after birth, before the known increase in mitochondrial biogenesis and switch from glucose to fatty acids as the chief energy substrate. PGC1 Alpha gene expression is activated by short-term fasting and overexpression of PGC1 Alpha increasing mitochondrial biogenesis and coupled oxygen consumption and ATP production. ERRalpha, NRF1 and NRF2 are key targets of the PGC1s in mitochondrial biogenesis. PGC-1 Alpha is highly hypersensitive to neuronal death due to oxidative stress and is an important protective molecule against ROS generation and damage. Antitumor antibiotics inhibit PGC-1 Alpha, leading to an increase in the generation of reactive oxygen species and decreased mitochondrial function as seen by decreased Complex-I / IV activities and ATP depletion (Kleeff et al., 2000),(M. Chen et al., 2015)). Regarding increase in oxidative stress, antitumor antibiotics reduce superoxide dismutase activity (Harada, Morooka, Ogawa, & Nishida, 2001). Increased ATP generation after mitochondrial biogenesis results in an increased oxygen demand that must be matched by a corresponding increase in oxygen supply. Moreover, overexpression of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha) increases the expression of a cohort of genes regulated by the dimeric hypoxia-inducible factor (HIF). Antitumor antibiotics have also shown to affect the HIF ((K. Lee et al., 2009),(Lou et al., 2010). Thus, PGC-1 coactivators offer a molecular signaling between

metabolic and immune pathways, decreasing the injurious effects of inflammation by decreasing the pro-inflammatory mediators and increasing the anti-inflammatory mediators. Therefore, antitumor antibiotics can increase the risk for neurodegeneration by affecting this pathway.

Table 4 b:

Markers	Effect of antibiotics (Doxorubicin, Actinomycin D, Bleomycin)
ROS	Doxorubicin increases ROS species (Kim et al; 2005) Bleomycin increases ROS species (Dayan et al; 2008). Actinomycin D increases ROS species (Kleeff et al; 2000).

PGC-1 Alpha	Doxorubicin inhibits PGC-1 Alpha (Guo et al; 2014).
NK-κB	Doxorubicin suppresses NK-κB expression (Chen et al; 2011).
HIF-alpha	Doxorubicin suppresses HIF-alpha expression (lee et al; 2009) Actinomycin D inhibits HIF alpha expression (Lou et al; 2010).
SOD2	Doxorubicin reduces SOD 2 levels (Sarvazyan et al; 1995) Actinomycin D decreases SOD2 levels (Harada et al; 2001).
Complex-I, IV	Doxorubicin decreases Complex-I, IV activity (Valls-Belles et al; 2010) Actinomycin D decreases Complex-I, IV activity (Patel et al; 2015).
ATP	Doxorubicin decreases ATP levels (Jeyaseelan et al; 1995)

TRAFs are defined by a unique C-terminal domain, the TRAF domain, that mediates interactions with many other signaling molecules, including apoptosis signal-regulating kinase NF-κB-inducing kinase (NIK), receptor-interacting protein (RIP), and TNF receptor-associated death domain protein (TRADD). Adaptor proteins, such as FADD (Fas-associated death domain) and TRADD (TNF receptor-associated death domain) are recruited to ligand-activated, oligomerized death receptors to mediate apoptotic signaling pathways. Apoptotic adaptor proteins regulate pro- and anti-apoptotic proteins following activation of the death receptors. Following association with the ligand-bound receptor, the adaptor proteins recruit pro-caspase-8 or pro-caspase-10 to form the DISC (death-inducing signaling complex). Formation of the DISC initiates a caspase signaling cascade that ultimately induces apoptosis. Caspase and PIDD (p53-induced protein with a death domain) are two adaptor proteins that associate with pro-caspase-2 to form the PIDDosome

following DNA damage. Formation of this complex leads to the cleavage and activation of caspase-2. DAXX (Fas death domain-associated-xx) and RIP1 (receptor interacting protein 1) are two additional adaptor proteins containing a death domain that are recruited to Fas and to TNF-RI respectively. These two proteins are involved in mediating caspase-independent apoptotic signaling pathways. Like TNF-RI and Fas, TRAIL receptors (TRAIL R1/DR4 or TRAIL R2/DR5) and DR3 also contain intracellular death domains and can induce apoptosis by way of adaptor-mediated caspase-8 or caspase-10 recruitment. TRAF-2 signaling subsequently leads to the activation of NF-κB which induces the expression of anti-apoptotic genes such as FLIP and Bcl-2. Regarding JNK, TRAF N-terminus promotes the recruitment of TRAFs to the membrane and this localization effect plays an important role in TRAF-mediated JNK activation. Antitumor antibiotics affect the TRAFF pathway leading to JNK activation (Strohm et al., 2002), increase pro-apoptotic protein expression, decrease antiapoptotic expression, increase cytochrome C release and increase caspase activity (Mungunsukh, Griffin, Lee, & Day, 2010a), (Ngok-Ngam, Watcharasit, Thiantanawat, & Satayavivad, 2013), (Kleeff et al., 2000). Thus, antitumor antibiotics can influence the JNK pathway and increase apoptosis leading to neurotoxicity.

Table 4 c:

Markers	Effect of antibiotics (Doxorubicin, Actinomycin D, Bleomycin)
JNK	Doxorubicin treatment induces c-Jun NH2-terminal kinase (JNK) activation. (Cho et al; 2013). Bleomycin increases activation of JNK (Yang et al; 2004). Actinomycin D Increases JNK activation (Strohm et al; 2002).

BAD/BAX	Doxrubicin treatment increases bax expression (NGOK-NGAM et al; 2013). Bleomycin treatment increases bax expression (Mungunsukh et al; 2010). Actinomycin D treatment increases bax expression (Brown et al;1996)
Bcl2	Doxorubicin decreases BCL2 expression (NGOK-NGAM et al; 2013). Bleomycin decreases BCL2 expression (Mungunsukh et al; 2010). Actinomycin D decreases BCL2 expression (Brown et al;1996)
Cytochrome-C	Doxorubicin increases cyt C (NGOK-NGAM et al; 2013) Bleomycin increases cyt C (Mungunsukh et al; 2010). Actinomycin D increases cytochrome c release (Brown et al;1996)
Caspases	Actinomycin D increases expression of caspase 3 (Kleeff et al; 2000). Doxorubicin increases expression of caspase-3 (Kim et al; 2005) Bleomycin increases expression of caspases (Mungunsukh et al; 2010).

2.5 Introduction to Topoisomerase inhibitors:

DNA is a double-stranded macromolecule in which each strand is a polymer of deoxyribonucleotide and the monomers that are linked by "phosphodiester bonds" between the 3' carbon of one deoxyribose molecule and the 5' carbon of another. Ester is a condensation product of an alcohol (sugars are "polyalcohols") and an acid (PO_4 3- is an acid). The term "diester" signifies the formation of two ester linkages by each phosphate molecule. DNA form supercoils, in which the helical axis of the DNA curves itself into a coil. The supercoil (or superhelix) structure is one physiological property of the problem of minimizing the excess energy that builds up when DNA molecules are deformed during the process of storage. Topoisomerases control DNA supercoiling by modifying the topological state of DNA and have been shown to be involved in

DNA replication and transcription. Topoisomerases control the topology of the supercoiled DNA double helix during the transcription of replicated cellular genetic materials. As the name suggests, Topoisomerase inhibitors are types of chemotherapeutics that inhibit the action of topoisomerases (topoisomerase I and II). During the process of chemo treatments, topoisomerases control the manipulation of the structure of DNA necessary for replication. There are two major types of topoisomerases, Topoisomerase I and II. Topoisomerase I initiates the cleavage of one strand of DNA molecule while Topoisomerase II cleaves both DNA strands. The partial or complete inhibition of this DNA replication mechanism results in the accumulation and stabilization of cleavable complexes and subsequent death of the cell. Another proposed model for the antitumor effect of Topoisomerase I inhibitors suggest that the accumulation or prolongation of Topoisomerase I cleavable complexes results in irreversible DNA replication defects and subsequent cell cycle arrest and cell death. Consequently, tumors that express significantly high levels of Topoisomerase I are presumed to be easy targets for Topoisomerase I inhibitors. The cytotoxic effect of Topoisomerase I inhibition depends on the length of exposure and not so much on the concentration of the Topoisomerase I inhibitor ((Mungunsukh, Griffin, Lee, & Day, 2010b), (A. Y. Chen & Liu, 1994)(Creemers, Lund, & Verweij, 1994).

a) Classification of Topoisomerase inhibitors: They are classified as topoisomerase I inhibitors and topoisomerase II inhibitors.

Topoisomerase I inhibitors (Irinotecan, topotecan): Topoisomerase I inhibitors are derivatives of the plant extract camptothecin. Irinotecan (CPT-11), a semi-synthetic derivative of camptothecin,

is approved in the United States for the treatment of colorectal cancer (Reginald B. Ewesuedoa and Mark J. Ratainb,1997). Topoisomerase I inhibitors are relatively a new class of anticancer agents. The mechanism of action of Ironotecan and topotecan are through the interruption of DNA replication in cancer cells which result in cell death.

Topoisomerase II inhibitors (Etoposide): Topoisomerase II is an enzyme that uses ATP to pass an intact helix through a transient double-stranded break in DNA to modulate DNA topology. Etoposide prevents topoisomerase II from restoring cleaved DNA. Etoposide thus converts topoisomerase II into a toxin that introduces high levels of transient protein-associated breaks in the genome of treated cells. Topoisomerase II exists as two highly homologous isoforms, alpha and beta, which differ in their production during the cell cycle (Kenneth R. Hande, 2008).

b) General mechanism of action of Topoisomerase inhibitors: Topoisomerase inhibitors are agents designed to interfere with the action of topoisomerase I and II. Topoisomerases are enzymes that control the changes in DNA tridimensional structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle. Inhibition of topoisomerases interferes with transcription and replication by causing DNA damage, inhibition of DNA replication, failure to repair strand breaks, and then cell death ((Creemers et al., 1994)).

c) Indication or Use of Topoisomerase inhibitors: Topotecan and etoposide are used in the treatment of colorectal, small-cell lung, ovarian and hematological cancers (S.A. Martin; 2016).

d) Adverse Drug Reactions / Side effects of Topoisomerase inhibitors: Ironotecan is known to cause chemotherapy induced diarrhea (Alexander et al; 2010). Irinotecan has been associated with non-specific dizziness and insomnia, as well as occasional episodes of dysarthria (Herbert B. Newton; 2012). Toxicities are generally hematologic, resulting in leukopenia and thrombocytopenia with an increased risk for infections and bleeding.

e) Hypersensitivity Reactions of Topoisomerase inhibitors: Hypersensitivity, anaphylaxis and acute fatal reactions are seen with intravenous administration of Ironotecan (Stan et al; 2011).

f) Effect of Topoisomerase inhibitors on CNS: Neurological complications can result in akathasia (irinotecan and prochlorperazine), insomnia and dizziness (Stan et al; 2011). Topotecan has shown to induce headache and lethargy. Irinotecan has been associated with nonspecific dizziness and insomnia as well as occasional episodes of dysarthria (Soffietti, Trevisan, & Rudà, 2014).

g) Molecular mechanisms involved with the cognitive impairment induced by Topoisomerase-inhibitors chemotherapeutics: Topoisomerase-inhibitors have shown to affect the IGF, PGC-1alpha and TRAFF signaling pathway, inducing neurotoxicity. Topoisomerase-inhibitors inhibit the IGF stimulation (Beppu, Nakamura, Linehan, Rapisarda, & Thiele, 2005), decrease Raf ((Wang, 2002)), Ras (Marengo et al; 2002), ERK1/2 (Boldt, Weidle, & Kolch, 2002),(M. Shin et al., 2016), Pi3K (Tsunetoh et al., 2010), AKT, (Boldt et al., 2002), (M. Shin et

al., 2016), (Tsunetoh et al., 2010), GSK3beta and mTOR(Tsunetoh et al., 2010). Insulin-like growth factor 2 (IGF2) is important in body growth and development. Insulin-like growth factor II significantly enhances memory retention and prevents forgetting and inhibitory avoidance. Learning also leads to an increase in hippocampal expression of IGF-II. Therefore, drugs affecting the IGF pathway can significantly lead to neurotoxicity.

Alterations of redox status, increase in reactive oxygen species (ROS) production, change in the levels of cytosolic calcium (Ca^{2+}), acetyl-CoA and pyrimidines can impact the energy biosynthetic pathways, cellular signal transduction pathways, transcription factors and chromatin structure to shift the cell from a quiescent, differentiated state to an actively proliferating one. The mitochondria control the regulation of the above signaling by affecting energy production, modulation of oxidation–reduction (redox) status, generation of reactive oxygen species (ROS), control of cytosolic calcium levels, contribution to cytosolic biosynthetic precursors such as acetyl-CoA and pyrimidines, and initiation of apoptosis through the activation of the mitochondrial permeability transition pore. Topoisomerase-inhibitor Chemotherapeutics have been shown to increase ROS and affect SOD activity (Timur et al; 2005, Khalife et; 2014, (M. Shin et al., 2016), affect PGC-1 alpha (Tsavaris et al., 2009), (M. Shin et al., 2016)affect mitochondrial respiration and decrease the production of ATP (Amani et al; 2015). Nuclear factor kappaB (NF-κB) is involved in T-cell activation and enhances HIV-1 gene expression. Nuclear factor kappaB is activated in response to numerous stimuli such as oxidative stress. Oxidative stress damages membrane lipids, proteins and nucleic acids. Topoisomerase inhibitors affect NF-kappaB (Piret

& Piette, 1996)Topoisomerase inhibitors suppress the expression of HIF-1alpha (Tsunetoh et al., 2010),(Beppu et al., 2005). Therefore, Topoisomerase inhibitors can cause neurotoxicity by affecting the mitochondrial function.

JNK signaling pathway (death pathway) controls cell death. Initially, c-Jun N-terminal kinase (JNK) activity was designated as ultraviolet- and oncogene-induced kinase activity on c-Jun. JNK kinases can be activated by numerous stimuli such as UV light, γ radiation, protein synthesis inhibitors, ceramide, DNA-damaging drugs, chemotherapeutics, TNF-α, and interleukins. The breakage in DNA is related to the activation of NF-κB, the secretion of TNF-α and an autocrine activation of the JNK pathway. The JNK pathway can be triggered by a breakdown (physical) in the DNA helix, leading to JNK activation and the resultant cellular consequences. Additionally, several cellular signaling ensues downstream of JNK activation. JNK activation can lead to cytoskeleton remodeling, the regulation of cell proliferation or apoptosis. Programmed cell death (apoptosis) is critical to numerous biological functions such as embryonic development, immune responses, tissue homeostasis and normal cell turnover. The origination and execution of apoptosis depend on activation of the receptor- and/or mitochondrial-dependent death pathways. Cells exposed to toxins or drugs can induce DNA mutation or damage. If, the damaged DNA cannot be instantly repaired, the cell programs itself to die (apoptosis) to avoid devious mutation or damage. There are two main downstream signaling of JNK pathway: one is the activation of death signaling such as c-Jun, Fos and apoptosis signaling such as BIM, BAD, BAX protein or active P53 transcription, to promote cell apoptosis; the other is the inhibition of the cell survival signaling such as STATs and CREB. Topoisomerase inhibitors affect the JNK expression (M. Shin et al.,

2016), increase pro-apoptotic protein expression (Creemers et al., 1994), decrease anti-apoptotic protein expression(Zhang et al., 2013), (Tomicic & Kaina, 2013), Khalife et; 2014, Perkins et al; 2000), increase cytochrome C release (Zhang et al., 2013), Perkins et al; 2000) and caspase activity(Zhang et al., 2013), (Tomicic & Kaina, 2013, Khalife et; 2014, (M. Shin et al., 2016). Therefore, topoisomerase inhibitors can affect the JNK pathway and induce apoptosis which can result in neurotoxicity.

Table 5 a:

Table 5 b:

Markers	Effect of topoisomerase inhibitors (Topotecan, etoposide)
ROS	Topotecan increases ROS species (Tunur et al; 2005, Khalife et; 2014) Etoposide increases ROS species (Shin et al; 2016).
IGF	Topotecan blocks IGF stimulation (Beppu et al., 2005)
Raf	Etoposide decreases Raf activation (Marengo et al; 2002)
PGC-1 Alpha	Etoposide increases Pgc-1 Alpha (Boldt et al., 2002) (M. Shin et al., 2016)
Ras	Etoposide decreases Ras activation (Marengo et al; 2002)
ERK1/2	Etoposide decresases ERK activation ((Boldt et al., 2002),(M. Shin et al., 2016)
NK-κB	Topotecan decreases expression of Nk-κB (Chaturvedi et al; 2016). Etoposide is known to decrease production of NK-κB (Yu et al, 2007, Calviño et al; 2015).
Pi3K	Topotecan inhibits Pi3K activation (Tsunetoh et al; 2010).
AKT	Etoposide decreases AKT activation Topotecan inhibits AKT activation (Tsunetoh et al; 2010).
HIF-alpha	Topotecan suppresses HIF-alpha expression (Tsunetoh et al., 2010)
SOD2	Etoposide increases SOD2 activity (Hempel et al; 2014)
Complex-I, IV	Etoposide decreases GSK-3beta expression (Li et al; 2007) Etoposide treatment reduces complex I and IV activity (Amani et al; 2015)
	Topotecan inhibits mTOR activation (Tsunetoh et al; 2010).
ATP	Etoposide treatment decreases ATP levels (Amani et al; 2015).

Table 5 c:

Markers	Effect of topoisomerase inhibitors (Topotecan, etoposide)
JNK	Etoposide decreases JNK activation (Shin et al; 2016).
BAD/BAX	Topotecan treatment increases bax expression (Khalife et al; 2014). Etoposide treatment increases bax expression (Perkins et al; 2000).
Bcl2	Topotecan decreases BCL2 expression (Zhang et al; 2013, Tomicic et al; 2009, Khalife et; 2014). Etoposide decreases BCL2 expression (Perkins et al; 2000).
Cytochrome-C	Topotecan increases cytochrome c release (Tomicic et al; 2009). Etoposide increases cytochrome c release (Perkins et al; 2000).
Caspases	Topotecan increases activation of caspase-9 and caspase-3 (Zhang et al; 2013, Tomicic et al; 2009, Khalife et; 2014). Etoposide increases expression of caspases (Shin et al; 2016).

2.6 Miscellaneous Chemotherapeutics:

Other classes of Chemotherapeutics include Antimicrotubule drugs (Estramustine), Retinoids (Bexarotene, Isotretinoin, Tretinoin), Enzymes (Asparaginase and Pegaspargase), Drugs that inhibit Ribonucleotide reductase (Hydroxyurea) and Drugs that inhibit Adrenocortical steroid (Mitotane) have been used as chemotherapeutics.

Microtubules are the key constituent of the cytoskeleton. Microtubules are rigid, hollow rods that constantly undergo assembly and disassembly within the cell. Microtubules help with determination of cell shape, motility (for some forms of cell locomotion), transport of organelles (intracellular) and separation of chromosomes during mitosis. They consist of α- and β-tubulin heterodimers, and the tubulin family of proteins is recognized as the target of the tubulin-binding chemotherapeutics, which suppress the dynamics of the mitotic spindle to cause mitotic arrest and cell death. Estramustine is an antimicrotubule drug and is mainly used to treat prostate cancer. Estramustine has shown to induce dizziness or faintness, speech problems, severe headache, sudden partial or complete loss of vision and severe hypersensitivity / allergic reactions. There are very few studies on the effect of Estramustine on cognition or memory.

Retinoids are chemicals that are structurally related to vitamin A. Physiologically, they regulate vision, cell growth (proliferation and differentiation), bone growth, immune function and activation of tumor suppressor genes. Retinoic acid is an active metabolite of vitamin A. Retinoic

acid has been used to treat lung, prostate, breast, ovarian, bladder, oral, and skin cancers. However, amongst the various retinoids, tretinoin is the most potent and widely used retinoid. Retinoids have shown to enhance memory and deficiency of retinoids have shown to facilitate pathogenesis associated with Alzheimer's disease (Zeng et al., 2017). There are also reports that show that Isotretinoin has shown to affect the IGF and TRAF pathway, which can affect the memory (Melnik, 2011), (Becker et al., 2016).

Asparaginase was initially obtained from *Escherichia coli* and *Erwinia carotovora*. It is mainly indicated in the treatment of acute lymphoblastic leukemia. Pegaspargase is a conjugate of a polyethylene glycol (PEG) moiety with the E. coli-derived asparaginase. With regard to the chemotherapeutic mechanisms of action, Asparaginase hydrolyzes L-asparagine to L-aspartic acid and ammonia. Asparagine is critical to protein synthesis in leukemic cells; some leukemic cells cannot synthesize this amino acid de novo due to the absence or deficiency of the enzyme asparagine synthase. Additionally, Asparaginase depletes asparagine, inhibits protein synthesis, arrests cell cycle in the G1 phase and induces apoptosis in susceptible leukemic cell populations. Due to the hypersensitivity reactions associated with Asparaginase, Pegaspargase was initially used to treat patients hypersensitive to native forms of L-asparaginase. U.S. Food and Drug Administration approved the use of pegaspargase (Oncaspar®, made by Enzon Pharmaceuticals, Inc.) for the first-line treatment of patients with acute lymphoblastic leukemia (ALL) as a component of a multi-agent chemotherapy regimen. As for the adverse effects, it causes hypersensitivity reactions, nausea and vomit, poor appetite, stomach pain and weakness, mouth sores, pancreatitis (pain in the upper abdomen that worsens with eating, swollen and tender

abdomen, nausea, vomiting, fever, and rapid pulse), hyperglycemia, hematological disorders (hemorrhage and thrombus formation), hyperbilirubinemia, and elevated transaminases. Regarding cognition, neurological problems occur due to bleeding and clotting in the CNS. The effects on the CNS are persistent headaches, epilepsy, and motor or cognitive deficiency .

Hydroxyurea is a multipotent drug and is used in the treatment of various diseases such as chronic myeloid leukemia, thrombocytosis, polycythemia vera, melanoma, head and neck cancer (used with radiation therapy), refractory ovarian cancer (ovarian cancer that has not responded or progressed after standard therapy). Regarding the pharmacological effects, when administered orally, hydroxyurea enters the cells by diffusion and is physiologically altered to a nitroxide radical. In the cytoplasm, hydroxyurea inactivates ribonucleotide reductase by suppressing the tyrosyl free radical at the active site of the M2 protein subunit. The entire replitase complex leads to the selective inhibition of DNA in S phase, resulting in cell death. Furthermore, chemical or radiation induced DNA damage is also reduced by hydroxyurea. Interestingly, the other pharmacodynamic effect of hydroxyurea is the escalation the level of fetal hemoglobin, leading to a reduction in the incidence of vasoocclusive crises in sickle cell anemia. It also reduces the level of episomal DNA and may potentially reduce drug resistance associated with duplicated genes retained as episomes in the treatment of cancer. Hydroxyurea exhibits synergistic effects with other chemotherapeutics. Hydroxyurea has synergistic effects with bleomycin. Synergy has also been observed between hydroxyurea and a number of other chemotherapeutic agents, including cytarabine and etoposide. Hydroxyurea renders cells sensitive to bleomycin because the suppressed tyrosyl free radical no longer stabilizes the adjacent iron center, making it more

susceptible to the chelating properties of bleomycin, which then produces active oxygen. Clinical and animal studies have shown that hydroxyurea can induce cognitive deficiency (de Montalembert et al., 1999),(Aygun et al., 2013). Regarding the molecular mechanisms of hydroxyurea associated with cognitive deficits, it decreases Ras, Raf, ERK, Pi3K, AKT, GSK-3beta and inhibits mTOR activation (Szymańska et al., n.d.). Hydroxyurea also affects JNK activation, increases BAX expression, decreases BCL2 expression, increases Cytochrome-C release and caspase-3 expression (Yan and Hales; 2008,("Toxic action of etoposide on mouse peritoneal macrophages and its modulation by interleukin 3. - PubMed - NCBI," n.d.)).

Corticosteroids (glucocorticoids and the mineralocorticoids) are produced in the adrenal glands. In humans, the major glucocorticoids are cortisol and corticosterone, and the major mineralocorticoid is aldosterone. In general, corticosteroids play an important role in carbohydrate & protein metabolism. Therefore, corticosteroids control serum glucose and maintain electrolyte & water balance. Glucocorticoids tend to cause the cells of the body to shift from carbohydrate catabolism to fat catabolism, accelerating the breakdown of proteins to amino acids and helping maintain normal blood pressure. Therefore, they alter the functions of the cardiovascular system, skeletal muscle, kidneys, and other organs. Stress, anxiety and other pathological conditions enhance the secretion of corticosteroids. Cushing's syndrome occurs due to prolonged secretion of corticosteroids. Increased glucocorticoids significantly increase the number of eosinophils, decrease lymphocytes, slow antibody formation and decrease the size of the thymus and the lymph nodes, leading to immunodeficiency. Aldosterone (mineralocorticoid) helps with the control of

sodium and potassium electrolyte balance. However, cortisol is less potent than aldosterone, causing sodium retention and potassium excretion. Mitotane, a derivative of the insecticide dichloro-diphenyl-dichloroethane decreases the production of hormones by inhibiting cells in the adrenal cortex. It is used to treat adrenocortical tumors (metastatic) without bone marrow depression. With regard to the side effects, Mitotane causes severe CNS damage, poor eyesight, confusion, drowsiness, depression, change in balance and speech and enlarged breasts. Cushing's disease patients treated with mitotane experience severe memory deficits (Schteingart et al., 1993).

Conclusion: Chemotherapy causes severe neurologically adverse effects affecting both the central and peripheral nervous system. CNS toxicities such as cognitive deficits, seizures, drowsiness, tremors, ataxia, other movement & mental disorders, or even comas (though uncommon) are commonly observed. Furthermore, patients with diabetes mellitus and hereditary neuropathies are more likely to develop peripheral neuropathy; therefore, these patients should be evaluated more carefully when potentially neurotoxic chemotherapy is administered. Thus, the proper assessment of central and peripheral neuropathy and its impact on quality of life should be carefully included in the chemotherapy regimens. Therefore, these adverse effects should always be taken into consideration when starting clinical chemotherapeutic trials in which higher doses or shorter intervals between doses are evaluated. Neuroprotective agents should ideally reduce the chemotherapy-induced neurotoxicity without reducing the antitumor effect. Unfortunately, data on neuroprotection in chemotherapy-related peripheral neurotoxicity are still controversial at this time. In our view, no neuroprotective agent can be recommended for standard use in daily clinical

practice. Therefore, the management of neurotoxicity mainly consists of the reduction of dosage of the cytostatic agent or longer intervals between cycles.

Chapter Two

Materials and Methods

3.0 Chemicals and Reagents:

Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin Solution and Trypsin-EDTA solution were purchased from ATCC. Thiazolyl Blue Tetrazolium Bromide (MTT) reagent was purchased from Tokyo Chemical Industry America. Phosphate buffer saline (PBS), Dimethylsulfoxide (DMSO), Nicotinamide adenine dinucleotide (NADH), 2`, 7-dichlorofluorescindiacetate (DCF-DA), Pyrogallol, Hydrogen Peroxide (H_2O_2),

Phosphoric acid, o-phthalaldehyde (OPA), L-Glutathione reduced, and Phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO). For protein quantification, Thermo Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL).

Rat dopaminergic neuron cells (N27):

DMEM medium with Fetal Bovine Serum (10%) and Penicillin-Streptomycin Solution (1%) was used to culture the N27 cells (Kovalevich & Langford, 2013). For MTT assay, N27 cells were cultured in 75 cm^2 flasks at 37°C and 5% CO_2. After the cells reached 80% confluency, they were detached by trypsinization and seeded in a 96 well plate at density of 1 x 10^5 cells/well. Cells were used within 14 passages (Zheng et al., 2014).

3.1 Treatment design:

Doxorubicin and cyclophosphamide being water soluble were dissolved in sterile water to make 1mM and 20mM stock solution respectively. Stock solutions were then diluted according to the experimental requirements. For the evaluation of neurotoxicity, six different concentrations of doxorubicin (1uM, 500nM, 100nM, 10nM, 1nM, 500pM) and cyclophosphamide (2mM, 200uM, 20uM, 2uM, 200nM, 20nM) were selected. N27 cells were exposed to different concentrations for 24 and 48 hours. To elucidate the mechanism of neurotoxicity due to chemotherapeutics, the N27 cells were exposed to 500nM and 500pM of doxorubicin and 2mM of cyclophosphamide for

24 hours. Each experiment was done by using freshly prepared drug solutions. MPP^+, a well-known exogenous neurotoxin served as the positive control.

3.2 Cytotoxicity Assay:

MTT cell viability assay was used to assess the cytotoxicity of the drugs. In MTT assay, the mitochondria of viable cells reduce the yellow coloured water soluble tetrazole reagent, MTT (3-(4, 5-dimethylthiazol-2- 31 yl)-2, 5-diphenyltetrazolium bromide) to an insoluble blue crystal formazan through succinate dehydrogenases. The resulted crystal formazan can be measured colorimetrically at 544nm (Berridge, Herst, & Tan, 2005; (Mosmann, 1983). After 24 and 48 hours incubation with different concentrations of doxorubicin, cyclophosphamide and MPP^+ in serum-fed and serum-free medium, 12 mM MTT stock solutions was prepared and then added in each well along with fresh culture medium. The plates were kept for 2 hours of incubation at 37°C and then the medium was aspirated. After which 200µl of DMSO was added to solubilize the formazan crystal and kept for 10 minutes. The absorbance was measured at 544 nm using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA). Results were expressed graphically as % cell viability. Cells were imaged using an Axiovert 25 inverted microscope equipped with a Nikon Coolpix 4500 camera (M. Zheng et al; 2014).

3.3 Protein quantification:

Protein quantification was done using Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

3.4 Quantifying Reactive Oxygen Species:

The reactive oxygen species was evaluated in the N27 rat dopaminergic cells treated with doxorubicin and cyclophosphamide by measuring the transformation of non-fluorescent chloromethyl-DCF-DA (2′, 7- 32 dichlorofluorescindiacetate, DCF-DA) to fluorescent DCF. ROS content was estimated spectrofluorometrically at excitation wavelength of 492 nm and emission wavelength of 527 nm. The control, doxorubicin and cyclophosphamide treated cell homogenates were incubated with 0.05% w/v solution of DCFDA in ethanol (10 µl), and phosphate buffer (150 µl) at 37°C for 1 hour. The fluorescent product DCF was measured using BioTek Synergy HT plate reader (BioTek, VT, USA). Results obtained were expressed as percentage change from the control (Dhanasekaran et al; 2008).

3.5 Catalase Activity

Catalase is an enzyme which is also an antioxidant that catalyses the breakdown of hydrogen peroxide into water and oxygen. This activity was measured by mixing the cell homogenate with PBS in the presence of 30mM of hydrogen peroxide. Breakdown of hydrogen peroxide was measured spectrophotometrically at 240nm for 1 minute. The decrease in absorbance was

observed and the enzyme activity was calculated as hydrogen peroxide decomposition/mg protein (Muralikrishnan & Mohanakumar; 1998).

3.6 Mitochondrial Complex-I Activity

Complex-I (NADH dehydrogenase) catalyses the oxidation of NADH to NAD^+ in and electron transport chain. The determination of NADH dehydrogenase activity was done spectrophotometrically at 340 nm, by mixing the cell homogenate with phosphate buffered saline and NADH. NADH oxidation was measured by the decrease in absorbance at 340 nm for 3 minutes. A standard curve was composed from commercially obtained NADH (Ramsay, Dadgar, Trevor, & Singer; 1986).

3.7 Mitochondrial complex IV activity

Complex IV assay was performed by mixing the cell homogenate, phosphate buffered saline and Cytochrome C. Activity of the Cytochrome C oxidase was measured spectrophotometrically at 550 nm. Change in absorbance for 3 minutes was used to determine the cytochrome C activity. A standard curve was created from commercially obtained Cytochrome C (Ramsay et al., 1986; Wharton & Tzagoloff; 1967).

3.8 Monoamine oxidase (MAO) activity

Monoamine oxidase is an enzyme responsible for metabolizing the monoamines. Activity of the total monoamine oxidase was measured fluorometrically by measuring the amount of 4-hydroxyquinoline generated due to the oxidation of kynuramine (Morinan & Garratt; 1985). MAO activity was calculated as 4-hyroxyquinoline formed/hour/mg protein (Albano, Muralikrishnan, & Ebadi; 2002, Muralikrishnan & Mohanakumar; 1998).

3.9 Nitrite assay

The final products of nitric oxide oxidation pathways are nitrite and nitrate, which is used as an expression of nitric oxide production. The nitrite assay was performed by using Griess reagent where in NO_2 react with sulfaniamide under acidic condition leading to production of diazonium ion. This diazonium ion association with N-(1-naphthyl) ethylene diamine to form 36 chromophoric azo product which can be measured spectrophotometrically at 545 nm (Giustarini, Dalle-Donne, Colombo, Milzani, & Rossi; 2008).

3.10 Superoxide Dismutase Activity

The autoxidation of pyrogallol in an alkaline environment results in the generation of superoxide anion radicals. Superoxide dismutase catalyses pyrogallol autoxidation inhibition can rapidly and conveniently be determined spectrophotometrically with visible light at 420 nm for 3 minutes by combining 2mM pyrogallol solution, 50 mM Tris buffer pH 8.2, and cell homogenate (Marklund & Marklund, 1974). Superoxide dismutase activity was measured as the change in absorbance at 420nm and expressed as percentage change from the control.

3.11 Glutathione content

When Glutathione (GSH) reacts with o-phthalaldehyde (OPT), fluorescence is produced which can be evaluated spectrofluorometrically (Cohn & Lyle, 1966). The assay samples contain cell homogenate, 0.1 M phosphoric acid, and 0.01 M phosphate buffer. First, mixing the cell homogenate with 0.1 M phosphoric acid to induce protein precipitate, then the samples were centrifuged at 12000 RPM for 10 minutes. Later, the supernatant was incubated with 0.1% OPT (dissolved in methanol) for 20 minutes at room temperature. Fluorometric readings were taken at an excitation wavelength of 340 nm and an emission wavelength of 420 nm. A GSH standard curve was prepared from commercially acquired GSH. The GSH content was calculated as of GSH/µg protein (Muralikrishnan & Mohanakumar, 1998; Y. Zheng et al., 2014).

3.12 Lipid peroxidation

Lipid peroxidation occurs due the oxidative breakdown of lipids, when ROS attack the polyunsaturated fatty acids in progression reaction process. Estimation of the lipid peroxidation content was done by quantifying malondialdehyde (MDA) content in the form of Thiobarbituric acid-reactive substances (TBARS) (Ohkawa, Ohishi, & Yagi, 1979). Control and designer drug treated cell homogenate (100µl) were incubated with ice cold 100µl Trichloroacetic acid (TCA, 20 % w/v), 400 µl Thiobarbuturic acid (TBA, 0.5 % w/v) and 500µl deionized water. Following the incubation, the samples were kept at 80ºC in water bath for 15 minutes. Centrifugation at 10,000 RPM was done after cooling for 5 minutes. The absorbance of the supernatant was

measured at 532 by using plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA). MDA levels were calculated as TBARS reactive substances per mg protein (Dhanasekaran et al., 2007; M. Zheng et al., 2014).

3.14 Glutathione peroxidase activity:

Glutathione peroxidase (GPx) catalyses the reduction of hydrogen peroxides and functions to protect the cell from oxidative damage. Oxidized glutathione (GSSG) is produced upon reduction of an organic hydrogen peroxide by GPx. GPx is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm. The rate of decrease in absorbance is directly proportional to GPx activity in the cell homogenate.

3.15 Quantitative RT-PCR

Total RNA was extracted from the N27 dopaminergic cells treated with high dose of cyclophosphamide (2mM), high dose of doxorubicin(500nM) and low dose of doxorubicin(500pM) by using the RNeasy Mini Kit (Qiagen; Valencia, CA). The quality and quantity of the total RNA were determined using NanoVue plus Spectrophotometer (GE Healthcare). Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen) and quantitative PCR was performed by using the QuantiTect SYBR Green Kit (Qiagen)

and iCycler iQ Real-Time PCR Detection System (Bio-Rad; Hercules, CA) according to the manufacturer's protocol.

3.12 Statistical Analysis

Data was reported as mean ± SEM. Statistical analysis were accomplished using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test (*$p < 0.05$ was considered to be statistically significant). Statistical analysis was performed using Prism-V software (La Jolla, CA, USA).

Chapter Three

4.0 Results

4.1 Doxorubicin and cyclophosphamide induced Dose-Dependent and Time-Dependent dopaminergic neurotoxicity (N27 neuronal death)

N27 Rat dopaminergic cells were treated with variable concentrations of doxorubicin (500pM, 1nM, 10nM 100nM, 500nM, 1000nM) for 24 and 48 and cyclophosphamide (0.02µM, 0.2µM, 2µM, 20µM, 200µM, 2mM) for 48 hours. Control cells were also subjected to that same environment and same volume of media. The well-known and established dopaminergic neurotoxin MPP+ was used as a positive control. Doxorubicin and cyclophosphamide caused significant dose-dependent and time-dependent of cell viability reduction comparing to control ($n=12$, $p<0.05$, Figure: 4.1 b, Figure: 4.1 c).

Treatment of N27 cells with cyclophosphamide for 24 hours did not show significant dose and time dependent cell death (data not shown). However after 48 hours there was a dose dependent decrease in cell viability. Even after 48 hours there was only 50-60% cell death at dose of (200uM-

2mM) due to cyclophosphamide (Figure: 4.1 a). The dopaminergic neurotoxin MPP+ induced dose dependent neurotoxicity (n=12, $p<0.05$, Figure: 4.1 d). Chemotherapeutics treatment showed some morphological changes in N27 neuronal cells. Cells treated with neurotoxins were structurally deformed, rounded, shrunken and also showed considerably less synaptic connections (Figure 4.1 e).

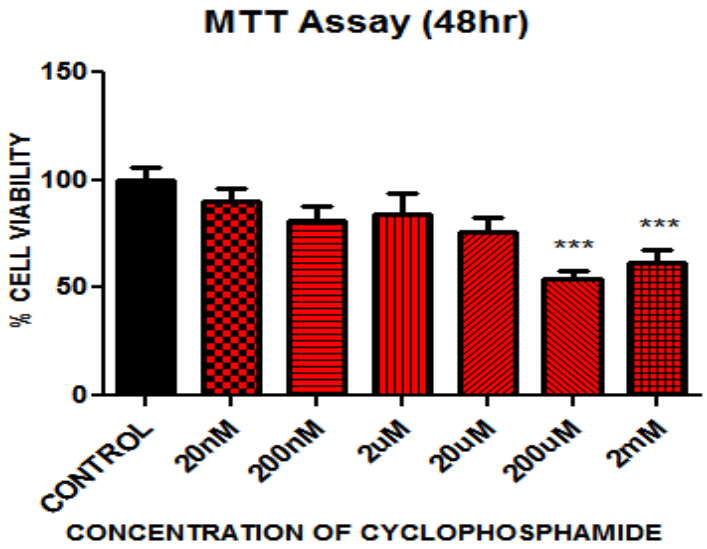


Figure: 4.1a) N27 dopaminergic Cells were treated with different doses of cyclophosphamide and incubated for 48 hours at 37°C. Cell viability was evaluated using MTT reduction assay (n=12). Results are expressed as percentage control ± SEM. Cyclophosphamide (200 and 2mM) significantly decreased the dopaminergic neuronal viability as compared to the control (n=12, $p<0.05$). Statistical comparisons were made using one-way ANOVA/Dunnett's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

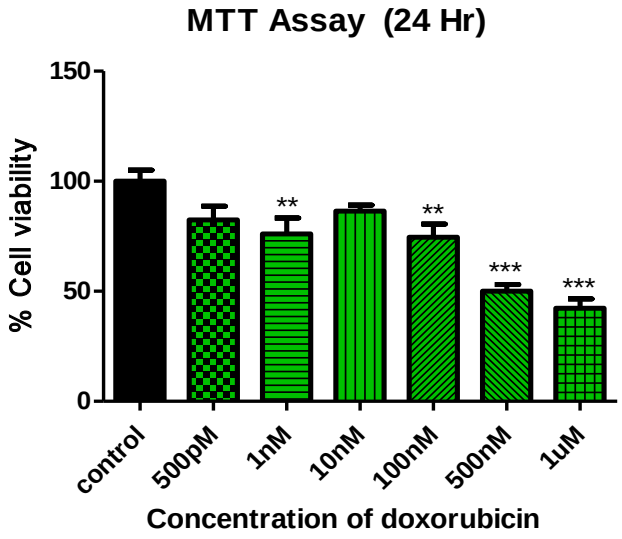


Figure 4.1 b) N27 dopaminergic Cells were treated with different doses of doxorubicin and incubated for 24 hours at 37°C. Cell viability was evaluated using MTT reduction assay (n=12). Results are expressed as percentage control ± SEM. Doxorubicin (1nM, 100nM, 200nM and 1µM) significantly decreased the dopaminergic neuronal viability as compared to the control (n=12, $p<0.05$). Statistical comparisons were made using one-way ANOVA/Dunnett's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

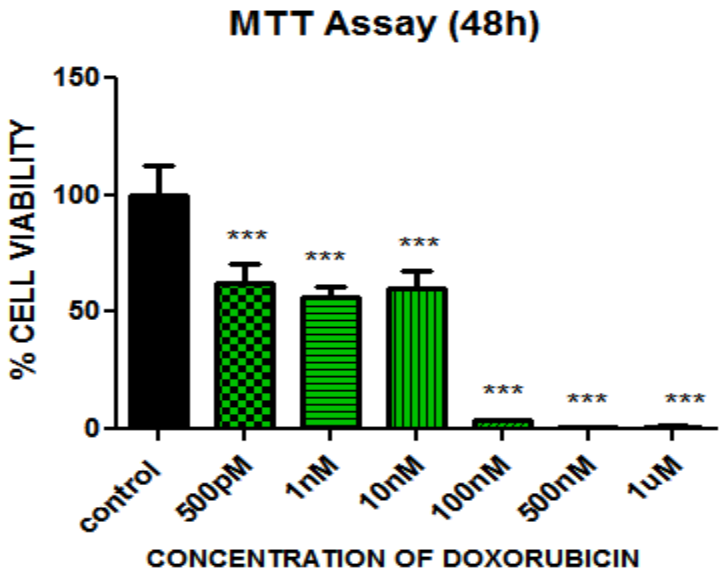


Figure 4.1 c) N27 dopaminergic Cells were treated with different doses of doxorubicin and incubated for 48 hours at 37°C. Cell viability was evaluated using MTT reduction assay (n=12). Results are expressed as percentage control ± SEM. Doxorubicin (500pM, 1nM, 100nM, 500nM and 1µM) significantly decreased the dopaminergic neuronal viability as compared to the control (n=12, $p<0.05$). Statistical comparisons were made using one-way ANOVA/Dunnett's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

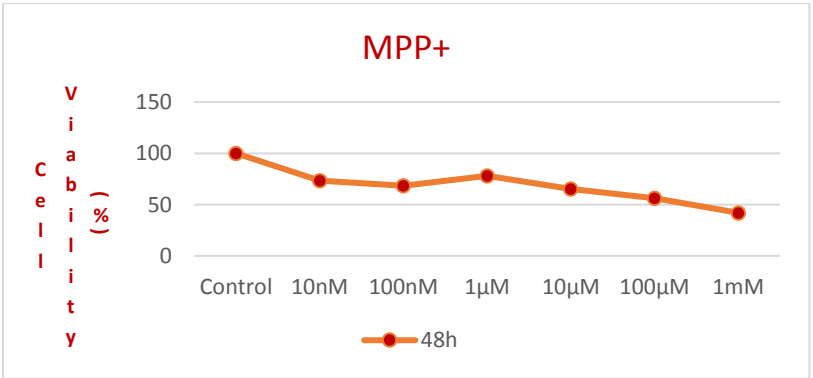


Figure 4.1 d) Cells were treated with different doses of MPP+ and incubated for 48 hours at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). Results are expressed as percentage control ± SEM. MPP+ dose dependently significantly decreased the dopaminergic neuronal viability as compared to the control (n=12, *$p<0.05$). Statistical comparisons were made using one-way ANOVA/Dunnett's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

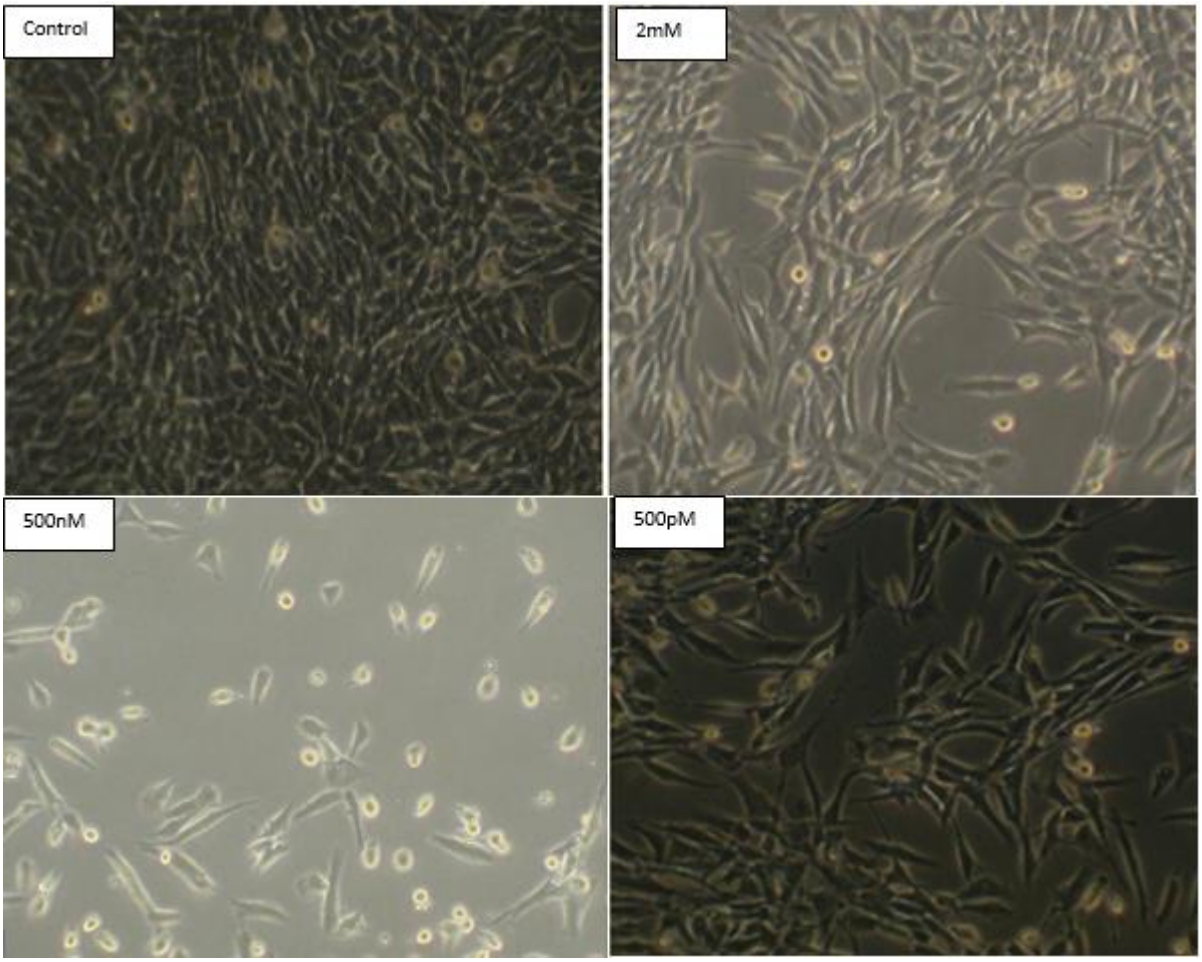


Figure 4.1d: Effect of doxorubicin and cyclophosphamide on N27 cells.

Morphological changes in N27 cells following the treatment with doxorubicin (500nM and 500pM) and cyclophosphamide (2mM) at 24 hours.

4.2 Doxorubicin and cyclophosphamide increases ROS generation

Diseases like aging, atherosclerosis, cancer and neurodegenerative disorders occurs mainly due to the generation of reactive oxygen species which in turn cause oxidative stress and this results in the death of healthy cells. Reactive oxygen species are known to destroy many biological molecules such as nucleic acids (DNS / RNA), protein and lipids which are required for the normal physiological functions (Dhanasekaran, Tharakan, & Manyam, 2008). Doxorubicin (500nM) and cyclophosphamide (2mM) dose-dependently increased ROS generation in N27 cells as compared to the control (n=5, $p < 0.05$; Figure 4.2).

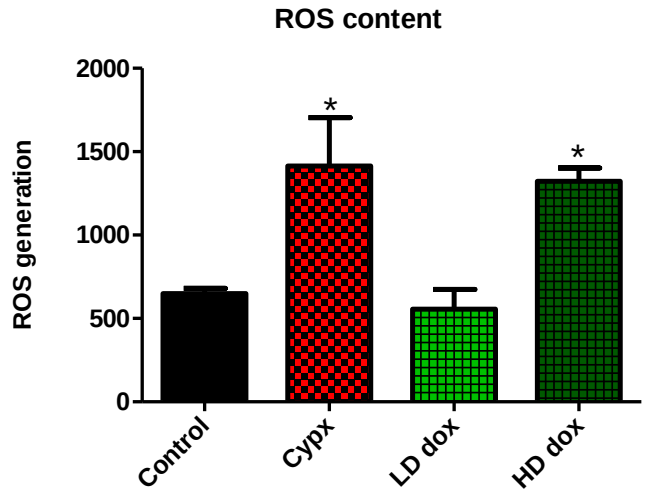


Figure 4.2: Effect of chemotherapeutics on ROS generation in N27 cells. Cyclophosphamide and doxorubicin causes oxidative stress by generating reactive oxygen species in N27 cells after 24 hours. The fluorescent product DCF was measured spectrofluorometrically. ROS was measured as relative fluorescence units (492/527 nm)/mg protein. Cyclophosphamide (2mM) and doxorubicin (500nM) showed a significant increase in ROS generation ($p < 0.05$, n=5). Results

are expressed as ROS generation control ± SEM. Statistical comparisons were made using oneway ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.3 Doxorubicin and cyclophosphamide increases nitrite content

Cyclophosphamide (2mM) and doxorubicin 500nM caused a significant increase in nitrite formation (n=5, $p<0.05$; Figure 4.3).

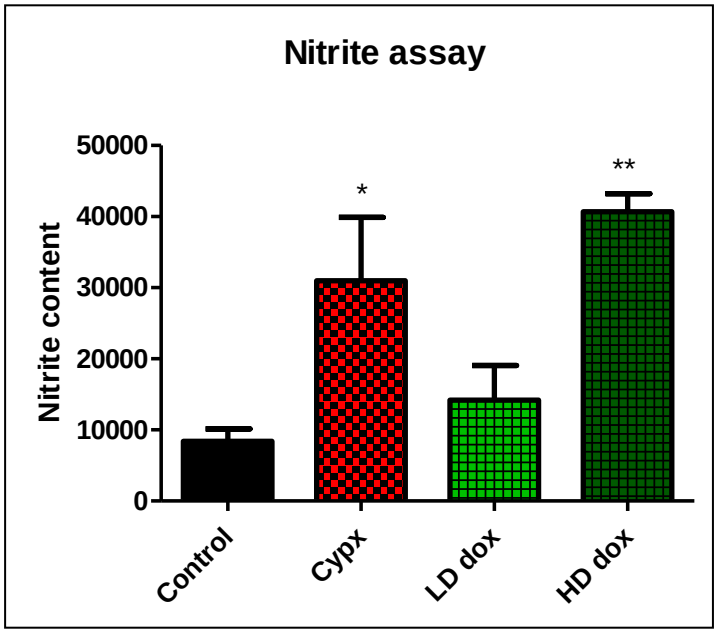


Figure 4.3: Effect of cyclophosphamide and doxorubicin on Nitrite production in N27 cells.

Nitrite production was determined spectrophotometrically at 540 nm. Results are expressed as Nitrite formed (nM/mg protein) control ± SEM. Both cyclophosphamide and doxorubicin caused a significant increase in nitrite production (n=5, $p<0.05$) in N27 cells after 24 hours incubation. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.4 Doxorubicin increases Glutathione peroxidase activity

Doxorubicin (500 nM) significantly increased the Glutathione peroxidase activity as compared to the control ($p < 0.05$, n=5, Figure-4.4). However, cyclophosphamide didn't show any significant increase in GSH-Px activity.

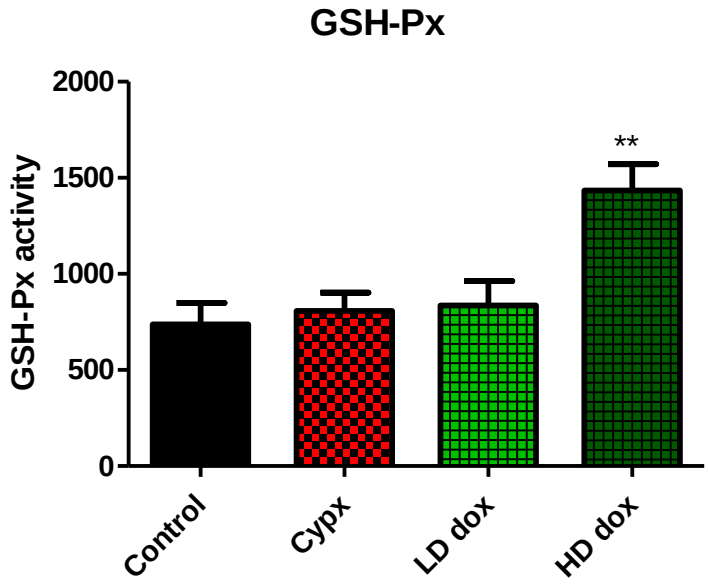


Figure 4.4 Effect of cyclophosphamide and doxorubicin on glutathione peroxidase activity in N27 cells. Glutathione peroxidase activity was measured spectrophotometrically using NADPH

as substrate. Doxorubicin (500nm) significantly increased the glutathione peroxidase activity as compared to the control (*p< 0.05, in = 5). Results are expressed as NADPH oxidized nM / mg total protein, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.5 Doxorubicin decreases Catalase activity

Catalase is an enzyme which catalyzes the breakdown of hydrogen peroxide into oxygen and water molecules. Doxorubicin (both doses) significantly decreased the catalase activity ($p < 0.05$, n=5, Figure-4.5). Cyclophosphamide exhibited a non-significant decrease in catalase activity.

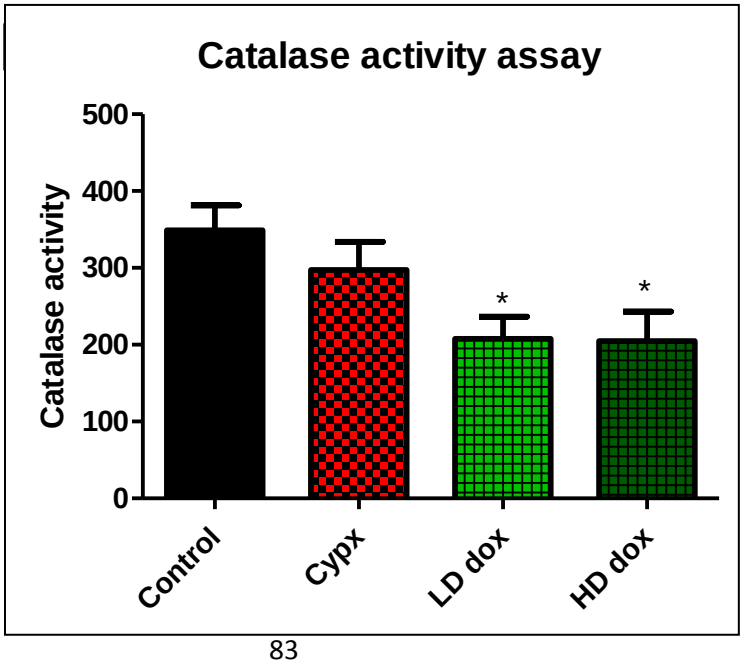


Figure 4.5 Effect of cyclophosphamide and doxorubicin on Catalase activity in N27 cells

Doxorubicin (both doses) significantly decreased the catalase activity ($p < 0.05$, n=5). Results are expressed as hydrogen peroxide oxidized uM / mg protein, Mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.6 Cyclophosphamide and doxorubicin increases Monoamine oxidase activity (MAO):

Monoamine oxidase metabolizes the monoamines like dopamine, serotonin and norepinephrine. Increase in the monoamine oxidase activity has shown to induce oxidative stress, apoptosis, glial activation, and decrease aggregated protein clearance. Hence, MAO activity has been connected to neurodegenerative diseases like Parkinson's disease and Alzheimer's disease (Siddiqui *et al;* 2010, Youdim and Lavie; 1994, Merad-Boudia, Nicole, Santiard-Baron, Saillé, & Ceballos-Picot, 1998). Doxorubicin significantly increased the monoamine oxidase activity dose-dependently as compared to the control (n=5, $p< 0.05$, Figure 4.6). However, cyclophosphamide had no effect on the monoamine oxidase activity.

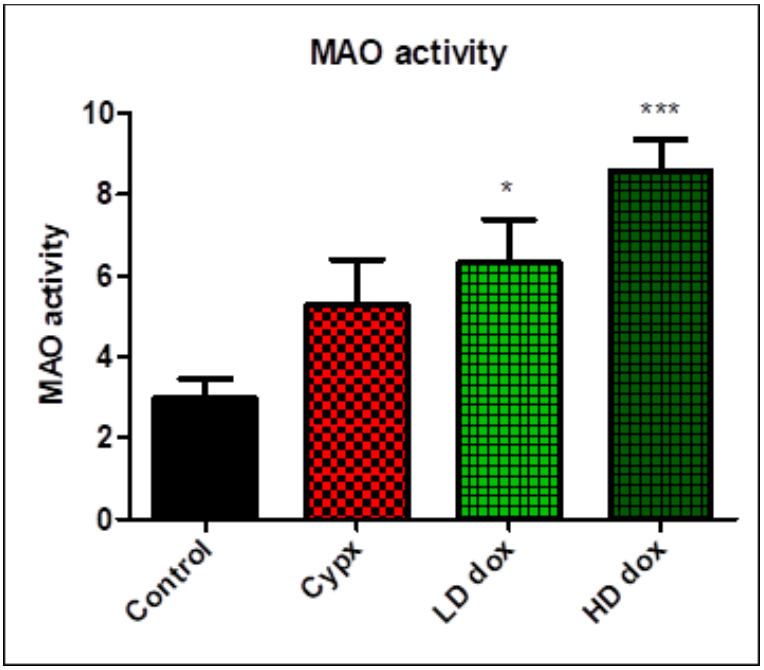


Figure 4.6: Effect of cyclophosphamide and doxorubicin on mitochondrial monoamine oxidase (MAO) activity in N27cells. Doxorubicin significantly increased the MAO activity in a dose-dependent manner (n=5, $p<0.05$) in N27 cells after 24 hours incubation. Total MAO activity was determined fluorimetrically at 315 nm excitation / 380 nm emission. Results are expressed as 4-Hydroxyquinoline formed uM/ mg protein, Mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.7 Mitochondrial Complex-I activity and Complex- IV activity:

Mitochondrial is considered as **a** regulator of cell viability because respiration of mitochondrial is responsible for production of energy (ATP). Many disorders like aging process and neurodegenerative disease such as Parkinson's disease, Alzheimer's disease and Huntington's disease are related to deficiency of Mitochondrial Complex-I and complex- IV. (Lin & Beal, 2006). Chemotherapeutics had no effect on the Complex-I and Complex-IV activity (Figure 4.7 a, b).

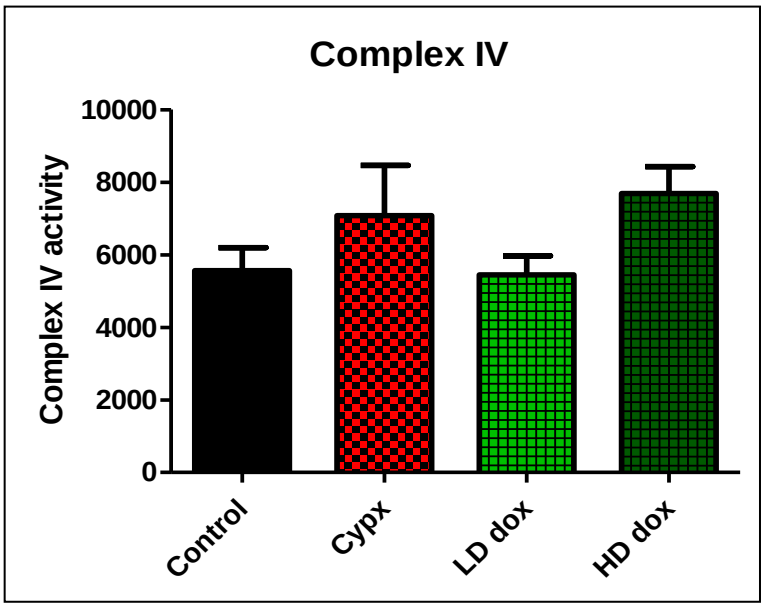


Figure 4.7 a): Effect of cyclophosphamide and doxorubicin on Mitochondrial Complex-IV activity in N27 cells. Complex-IV activity was measured colorimetrically using cytochrome-C as substrate. Doxorubicin and cyclophosphamide did not have any significant effect on the mitochondrial Complex-IV activity (n = 5). Results are expressed as cytochrome c oxidized (μM)/mg protein, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

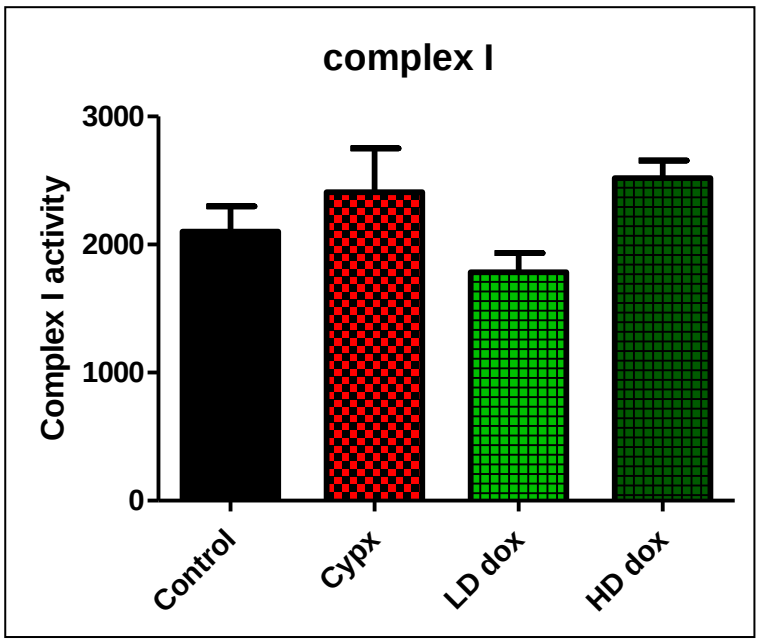


Figure 4.7 b): Effect of cyclophosphamide and doxorubicin on Mitochondrial Complex-I activity in N27 cells. Complex-I activity was measured spectrophotometrically using NADH as substrate. Doxorubicin and cyclophosphamide did not have any significant effect on Complex-I activity. Results are expressed as NADH oxidized (μM)/mg protein, mean ± SEM.

4.8 Doxorubicin and cyclophosphamide increases Superoxide dismutase activity:

Superoxide dismutase is an enzyme known to dismutase the superoxide radicals or in simple terms known to scavenge the harmful radicals. Cyclophosphamide and doxorubicin (500 nM) significantly increased the superoxide dismutase activity as compared to the control (n=5, $p < 0.05$, Figure 4.8).

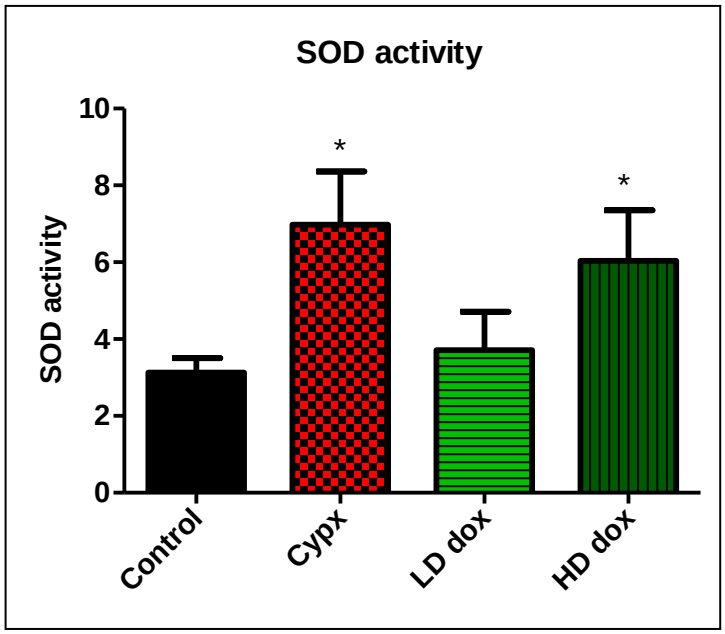


Figure 4.8: Effect of cyclophosphamide and doxorubicin on SOD activity in N27 cells. SOD activity was measured spectrophotometrically using pyrogallol as a substrate. High dose (500nm) of doxorubicin and cyclophosphamide increased SOD activity significantly (*$p < 0.05$, $n = 5$). Results are expressed as inhibition of pyrogallol autoxidation/mg total protein, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.9 Doxorubicin increases Lipid peroxide formation

Lipid peroxidation is a chain reaction mechanism in which reactive oxygen species attack polyunsaturated fatty acids resulting in the oxidative degradation of lipids. Lipid peroxidation was estimated by measuring the amount of thiobarbituric acid-reactive substances (Ohkawa et al., 1979, Zheng et al., 2014). Doxorubicin (500 nM) significantly increased the lipid peroxidastion as compared to the control ($n=5$, $p < 0.05$, Figure 4.9). However, cyclophosphamide had no effect on the monoamine oxidase activity.

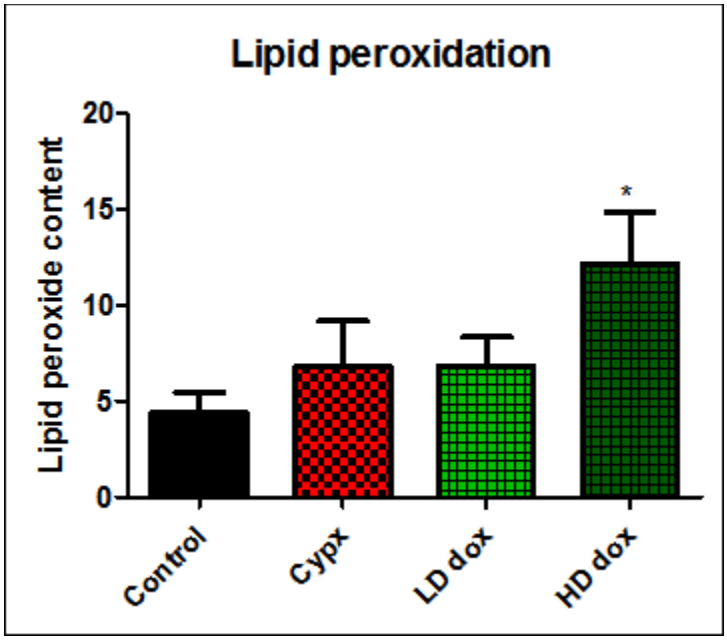


Figure 4.9 Effect of cyclophosphamide and doxorubicin on N27 cells for lipid peroxide formation. Lipid peroxide was measured spectrophotometrically. Due to the increased ROS generation, of doxorubicin (500nM) induced a significant formation of lipid peroxide (*$p < 0.05$, $n = 5$). Lipid peroxide formation was measured as TBARS formed (532 nm)/mg protein and the result are expressed as lipid peroxide formed as compared to the control. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.10 Cyclophosphamide and doxorubicin decreases Glutathione content

Doxorubicin (500 pM and 500 nM) significantly decreased the glutathione content as compared to the control (n=5, p< 0.05, Figure-4.10). However, cyclophosphamide had no effect on the glutathione content.

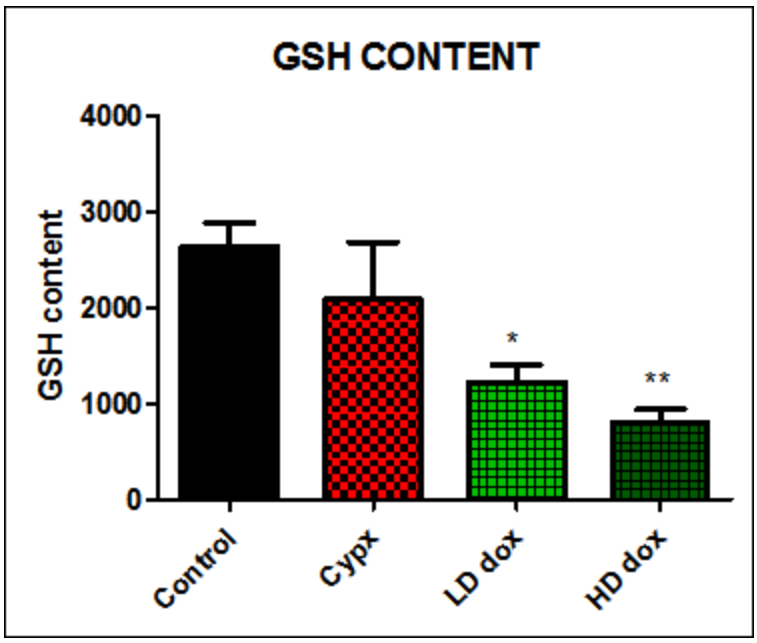


Figure 4.10 Effect of cyclophosphamide and doxorubicin on N27 cells on glutathione content: Glutathione content was measured spectrofluorimetrically. Low dose and high dose of doxorubicin showed significant depletion in GSH content (*$p < 0.05$, n = 5). Results are expressed as GSH content (μM)/mg protein, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

4.11 Cyclophosphamide and doxorubicin increases BAX expression and MAO-B expression:

RT-PCR showed significant increase in MAO-B expression by high dose of doxorubicin. With regards to the apoptotic marker, BAX expression was increased by cyclophosphamide and high dose of doxorubicin significantly as compared to the control (n=3, *p< 0.05, Figure-4.11 a, b).

Figure 4.11 Effect of chemotherapeutics on MAO-B and BAX Gene Induction in N27 cells.

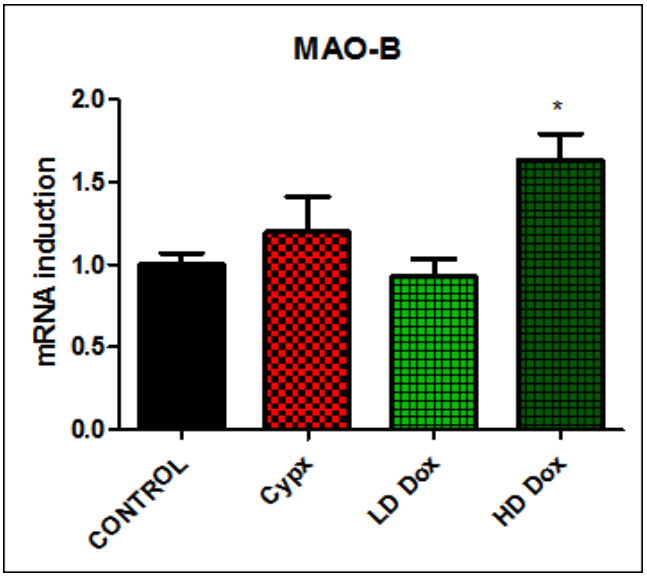


Figure 4.11 a: Effect of cyclophosphamide and doxorubicin on N27 cells on MAO-B expression: Doxorubicin (500nM) showed a significant increase in MAO-B expression in N27 cells compared to the control (n = 3. *, p < 0.05). Significance was measured using GAPDH control. Statistical

comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

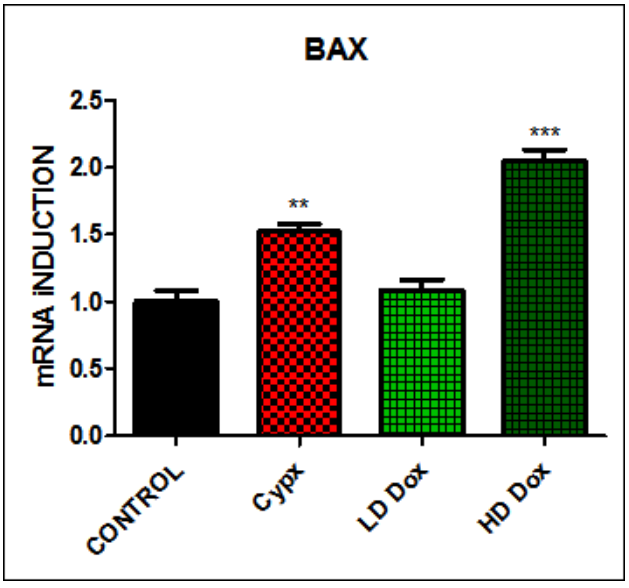


Figure 4.11 b: Doxorubicin (500nM) and cyclophosphamide (2mM) showed a significant increase in BAX expression in N27 cells compared to control (n = 3. *, $p < 0.05$). Significance was measured using GAPDH control. Statistical comparisons were made using one-way ANOVA/Dunnet's multiple comparison test. Note (*) indicates a statistically significant difference when compared to controls.

5.0 Discussion:

Parkinson's disease (PD) is a common neurodegenerative disease which results mainly from the death of dopaminergic neurons in the substantia nigra. Exposure to neurotoxins and excessive metabolism of dopoamine leads to generation of hydrogen peroxide which in turn produces ROS (Graham, 1978). The most common dopaminergic endogenous and exogenous neurotoxins are hydrogen peroxide, divalent metals, 6-hydroxydopamine (6-OHDA), 1-

methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and herbicides (paraquat / diquat). Some of these neurotoxins are structurally similar to monoamines like dopamine and norepinephrine. These neurotoxins exhibit a strong affinity for several catecholaminergic plasma membrane transporters such as the dopamine (DAT) and norepinephrine transporters (NET). Hence, these neurotoxins can enter monoaminergic (both dopaminergic and noradrenergic) neurons to cause damage to the both the peripheral and the central nervous systems. Its mode of action is by producing a bilateral catecholaminergic lesion which depletes the dopamine level. These dopaminergic neurotoxins induce oxidative stress (generation of reactive oxygen species and quinones, depletion of antioxidants and increase in proxidants), mitochondrial dysfunction (inhibition of Complex-I and Complex-IV activity, depletion of ATP) and apoptosis (increase in proapoptotic and decrease antiapoptotic markers (Yu Watanabe et al; 2016). These dopaminergic neurotoxins that produces parkinsonian like syndrome like tremor, rigidity, slowness of movement, postural instability, and even freezing. Most of the dopaminergic neurotoxins readily crosses the blood brain-barrier and can interact with enzymes associated with dopamine metabolism (tyrosine hydroxylase, COMT, MAO-B). These neurotoxins also cross many cellular membranes to accumulate in organelles such as mitochondria which impairs oxidative phosphorylation by inhibiting reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase activity. They also inhibits the formation of microtubules from tubulin which causes neurodegeneration because excess of tubulin monomers is considered toxic to cells (Burke D, Gasdaska P, Hartwell L; 1989, McCormack AL; 2002).

Chemotherapeutics-induced neurotoxicity has been reported to increase significantly around the world (Yoon et al., 2007)(Beinert et al., 2000)(Snyder et al., 2018)(Banach, Juranek, & Zygulska, 2017). Therapeutic use of chemotherapeutic drugs has shown to decrease cancer, however, there are numerous adverse effects associated with their use. General neurotoxicity of chemotherapeutics includes mental depression, alterations in consciousness, seizures, cerebral infarctions, paralysis, peripheral neuropathy, leukoencephalopathy, ototoxicity cerebral infarctions, leukoencephalopathy and cognitive impairments (Beinert et al., 2000). Furthermore, Chemotherapeutics also causes changes in ion channels on dorsal root ganglia and dorsal horn neurons that can lead neuropathy (Kukkar, Singh, & Jaggi, 2013). Many chemotherapeutics are known to treat brain tumors like cisplatin, carmustine, ifosfamide because they are known to cross blood brain barrier. However, doxorubicin does not readily cross the blood brain barrier but regarding its therapeutic use, novel and improved new drug delivery system enhances the delivery of doxorubicin in the CNS to treat brain tumors (Belhadj et al., 2017),(Li et al., 2014), (Niu, Wang, Ke, & Zheng, 2014)(Gong et al., 2011). Ondansetron (5-HT3 antagonist) is usually prescribed with doxorubicin to treat nausea and vomiting. Interestingly, Ondansetron is known to block the P-Glycoprotein pump which facilitates the entry of doxorubicin into the CNS (Sardi et al., 2014).

Chemotherapeutics have significantly shown to impair cognitive performance (Winocur, Vardy, Binns, Kerr, & Tannock, 2006)(Rzeski et al., 2004). Hippocampus and cortex mainly deal with cognition. Vitamin B12 and folate deficiency is very common side effect of chemotherapy which is said to have a role in induction abnormal Tau phosphorylation and amyloid precursor protein upregulation**,** which can lead to the pathogenesis of Alzheimer's disease (Yoon et al., 2007). Recent studies suggest that the cognitive symptoms are generally subtle and often improve after completion of treatment (Schagen & Wefel, 2013)(Janelsins, Kesler, Ahles, & Morrow, 2014). However, cognitive impairment may have a substantial impact on the quality of life and on a survivor's ability to function daily task. Thera are few reports which positively correlates with the dose regimes of chemotherapy ((Yoon et al., 2007)(Beinert et al., 2000)(Snyder et al., 2018)). These cognitive defects may persist more than 20 years after chemotherapy with cyclophosphamide, methotrexate, and fluorouracil (Koppelmans et al., 2012). Chemotherapeutics have shown to decrease neurogenesis, induce oxidative stress, mitochondrial dysfunction and apoptosis in hippocampus and cortex (Yoon et al., 2007)(Beinert et al., 2000)(Snyder et al., 2018)(Banach et al., 2017), (An et al., 2011),(Gaman et al., 2016b). The development of cognitive impairment can due to various mechanisms including the original underlying cancer, cancer therapy, aging, psychoactive medications, psychosocial, environmental and genetic risk factors. Consequently, this is an essential area of research given its clinical importance. Currently effective interventions for patients with cancer with CRCI are also lacking, as are guidelines on how to care for older patients with cancer and CRCI.

Thus, Chemotherapeutics related Cognitive impairment (CRCI) has become a hot topic for the researchers now. Therefore, there is an urgent need to find the specific underlying neurotoxic mechanisms which can in aid in development of the subsequent effective therapy with minimal adverse effects and hypersensitivity reactions to reduce the cognitive impairment. To our knowledge, our study is the first report that doxorubicin and cyclophosphamide can cause dopaminergic toxicity leading to disorders like Parkinson's. Doxorubicin and cyclophosphamide dose dependently induced cell dopaminergic neuronal cell death which we compared with the exogenous neurotoxin MPP+ as a positive control. Previous studies showed that doxorubicin and cyclophosphamide induce oxidative stress, apoptosis and mitochondrial functions (Kim et al; 2005, Amelia et al; 2016). Hence, we evaluated the neurotoxic effect of cyclophosphamide and doxorubicin on N27 dopaminergic cell lines. Furthermore, we also elucidated the neurotoxic mechanisms related to oxidative stress, mitochondrial functions and apoptosis. In the current study, chemotherapeutics (doxorubicin and cyclophosphamide) induced oxidative stress and apoptosis (increased BAX expression) in the dopaminergic neurons without affecting the mitochondrial functions. With regards to oxidative stress, cyclophosphamide and doxorubicin increased the generation of ROS and nitrite content resulting in lipid peroxidation. Cyclophosphamide and doxorubicin depleted glutathione, decreased catalase activity, increased glutathione peroxidase, SOD and MAO activity.

Thus, our *in vitro* study in the dopaminergic neurons suggest that the prolonged and chronic use of Cyclophosphamide and doxorubicin can potentially lead to noteworthy dopaminergic neurodegeneration resulting movement disorders like tremor, dystonia and Parkinson's disease.

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