GENOTOXIC EFFECTS OF A NEW ORGANOTIN COMPOUND $Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} IN MAMMALIAN SYSTEM

BY

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Declaration

I, Christine Syng-ai, do hereby declare that the subject matter of this thesis is a record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other university/institute.

This is being submitted to the North Eastern Hill University for the degree of Doctor of Philosophy in Zoology.

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Christine

Contents

Introduction

Since its discovery in 1963, (Frust *et al*, 1993) metal chelation continues to play an important role in the cure and cause of malignancy. One of the most outstanding recent developments in the field of metal compounds in medicine was Rosenberg's accidental discovery that platinum complexes possess anti-tumor activity (Rosenberg, 1969). The most widely used chemotherapeutic regimen includes platinum compounds. (McGuire *et al* 1993) However, drug resistance of tumor cells (Johnson *et al* 1993), toxicity (nephrotoxicity, ototoxicity, neurotoxicity) and side effects (emesis) frequently limit the clinical usefulness of cisplatin. Chemical modifications of the drug have been carried out to reduce toxic effects and improve their antitumor efficacy and pharmacokinetic properties (Perez 1991). Many cisplatin analogs have been investigated in recent years (Carnetta *et al* 1996). Moreover, several non-platinum metal complexes have been extensively investigated in recent years (Moebus *et al* 1997) and antitumor activity has been found in derivatives of rhodium, indium and palladium and other compounds containing copper, gallium, titanium, tin and germanium were tested for their biological activity in human cancer cells (Keppler 1993, Gielen 1990, Respondek *et al* 1996.).

Organotin compounds show a spectrum of biological effects and have been extensively studied and used commercially as fungicides, bactericides, acaricides and wood preservatives (Blunden *et al* 1985), anti-fouling coating, insecticides and plastics stabilizers (Sasaki *et al* 1993). The biological effects of organotins have been well investigated (Boyer 1989), these include skin toxicity, neurotoxicity, immunotoxicity, and carcinogenicity (Innes, 1969). Organotins have also been widely studied as environmental pollutants in both land and water. Triphenyltin have been extensively investigated as a major seawater pollutant in Japan (The Environment Agency of Japan, 1991). But there is virtually no report on the interaction of organotin compounds with the DNA. Early studies showed that they are inactive towards transplanted mouse cancers (Krause 1969). However in 1972, Brown demonstrated that tryphenyltin acetate, but not chloride, when

administered in the food or by injection retarded tumor growth in mice. Only scanty and scattered information is available on their activity against cancer (Evans *et al* 1985; Saxena 1987).

The development of cancer is no longer a mystery. During the past two decades, investigators have made astonishing progress in the deepest bases of the process - those at the molecular level, but translation of new understanding into clinical practice is complicated, slow and expensive. The term "cancer" refers to more than 100 forms of the disease. Almost every tissue in the body can spawn malignancies some even yield several types. What is more, each cancer has unique features. The 30 trillion cells of the normal, healthy human body live in a complex, interdependent condominium, regulating one another's proliferation. Indeed, normal cells reproduce only when instructed to do so by other cells in their vicinity. Such unceasing collaboration ensures that each tissue maintains a size and architecture appropriate to the body's need. Cancer cells, in stark contrast, violate this scheme; they become deaf to the usual controls on proliferation and follow their own internal agenda for reproduction. They also possess an even more insidious property - the ability to migrate from the site where they began, invading nearby tissues and forming masses at distant sites in the body. Tumors composed of such malignant cells become more and more aggressive over time, and they become lethal when they disrupt the tissues and organs needed for the survival of the organism as a whole.

Over the past 25 years, scientists have uncovered a set of basic principles that govern the development of cancer. We now know that the cells in a tumor descend from a common ancestral cell that at one point - usually decades before a tumor becomes palpable - initiated a program of inappropriate reproduction. Further the malignant transformation of a cell comes about through the accumulation of mutations in specific classes of the genes within it. These genes provide the key to understanding the processes at the root of human cancer. Two classes of gene, which together constitute only a small proportion of the full genetic set, play major roles in triggering

cancer. In their normal configuration, they choreograph the life cycle of the cell - the intricate sequence of events by which a cell enlarges and divides. Proto-oncogenes encourage such growth, whereas, Tumor suppressor genes inhibit it. Collectively these two gene classes account for much of the uncontrolled cell proliferation seen in human cancers (Weinberg1996).

The history of chemotherapy is rather unusual. Although some Egyptian writings from around 1600 B.C. described the use of crude drugs to treat ulcerating skin tumors, and the first documented case of cancer chemotherapy dates back to 1865 (Lissauer 1865), modern cancer chemotherapy is only about five decades old. Sulfur mustard, a toxic chemical was developed during World War I for used by the army in the battlefield. It caused burns in the eyes, on the skin and in the respiratory tract of the soldiers exposed to it and incapacitated them. As early as 1931, attempts were made at using sulfur mustard for treating squamous carcinomas in humans, but it was too toxic for systemic use (Adiar and Bagg 1931) It was several years before Gilman and colleagues discovered that nitrogen mustard inhibited the growth lymphosarcoma in mice, and later initiated the first clinical trial of nitrogen mustard on patients with lymphosarcomas (Gilman and Philips 1946). It was not until after World War II that Gilman's work were published (Gilman and Philips 1946), marking the beginning of modern day cancer chemotherapy. A derivative of this compound, nitrogen mustard, was made later. It had anti-tumor properties. Its first trial on patients was done in 1942. Since then hundreds of thousands of chemicals have been synthesized and tested for anti-cancer action.

There are two strategies for the development of new anti-tumor drugs. The first is to search for agents that exert cytotoxicity (Kanzawa *et al* 1990, Kanzawa *et al* 1995), and second is to screen new agents for their effectiveness against tumor cells (Kanzawa 1981, Horichi *et al* 1990, Ohmori *et al* 1990). The type of chemicals tested seems to come from every possible source. Most are synthesized in laboratories. In addition, natural products isolated from plants and animals, antibodies made by organisms from

different habitats and products mentioned in various indigenous systems of medicine undergo rigorous testing. Only a handful of them make it to the final stage of treating patients. Thus, finding a new effective anti-cancer drug is an extremely expensive task.

The effect of a new compound is first tested on mouse tumors. If it kills these tumors cells, it has then to undergo tests for toxic effects on different systems. In this process it is often necessary to change the structure of the original molecule so that it can kill cancer cells but not have undesired effect on the rest of the body. The entire process may take a very long time, up to ten years from the first tests in mice to the time the drug comes, for what is called, phase one trial.

Certain advantages in the chemotherapy of infectious diseases (bacterial, fungal or parasitic) do not exist to aid in the treatment of cancer. Most important, the concept of selective toxicity is usually not operative. The often-significant biochemical differences between the cells of the host and the invading organism simply do not exist between the cancerous cells and normal cells of the same tissue. The great majority of anticancer drugs bring about a general nonselective interference in cellular processes and cell growth. In fact, many of these cytotoxic agents are almost equally detrimental to normal and neoplastic tissues. Therefore it is not surprising that these drugs produce serious and often debilitating side effects. The usually high doses of these cytotoxic agents that are needed to arrest tumor growth or to obtain a temporary remission of symptoms will also depress bone marrow function; causing drastic reductions in the number of platelets, leukocytes and lymphocytes, which in turn lead to greater susceptibility to infection and internal bleeding. One of the basic tenets of cancer chemotherapy is that all neoplastic cells be eradicated, if a cure is to be achieved. Whether the modality used is surgery, radiation or chemotherapy, or most often a combination of these, total cell kill is the objective of treatment (Gringauz, 1997).

Many organotin compounds have been tested by the United States' National Cancer Institute. Surprisingly and interestingly enough, the percentage of active compound against P-388 lymphocytic leukemia in mice was indeed very high, up to almost 50% for those complexes, which contain diorganotin - dihalides (Gielen 1986). During the period 1973-1977, the Institute for Organic Chemistry TNO, Utrecht, The Netherlands carried out a wide variety of tests on organotin compounds including anti-tumor activity, some 115 complexes were found to exhibit reproducible activity against P-388 lymphocytic leukemia in mice. The screenings results indicate that diethyltin dihalide adducts tend to show the highest activity (Crowe1987).
A consideration of the screening results for a series of complexes (where R=Cn, n=1-6, X=Cl, Br, I) reveals that many more dibromo-complexes are active than dichloro- or diiodo- compounds, whilst for R the diethyl- and / or diphenyl- tin complexes usually possess the highest activity. However, no real link between the acceptor strength of the parent organotin halide and activity can be discerned (Crowe 1984). The majority of the ligands used were bidentate to ensure that the resulting octahedral complex possessed cis-halogens, which has been shown in the case of platinum compounds, to be an essential requirement for activity (Cleare 1974).

Another study of metallocene dichloride (VI) has shown that the anti-tumor activity of such compounds, as well as that of cisplatin, may be dependent upon the Cl-M-Cl bond angle and hence the corresponding non bonding Cl---Cl distance (bite). Only those compounds for which the Cl-M-Cl angle is <95°, giving a bite size of <3.6A° are active (Kopf *et al* 1983). But the X-ray structural parameters of some of the diorganotin dihalide complexes show that the Cl-M-Cl bond angles of both active and inactive compounds are all of the same magnitude. This suggested that the mode of action for the formation of metal-base cross-links for the organotin depends more on the Sn-N bond lengths rather than the Cl-M-Cl bond angle (Crowe 1984) The structure / activity relationship for diorganotin-dihalide complexes is that the Sn-N bond lengths appear to determine the anti-tumor activity

(Crowe 1984). The more stable complexes exhibit lower activity. Those with an average Sn-N bond length larger than 2.39A° are active, whereas those with bond length lesser than 2.39A° are inactive. This implies that a predissociation of the (bidentate) nitrogenous ligand might be a crucial step in the formation of tin-DNA complex (Gielen 1986).

The mode of action of cisplatin and its analogues in their anti-tumor activity appears to be fairly well established; the X-ray structure clearly shows that cisplatin cross-links the DNA and that the binding sites are the N-7 nitrogen atom on two adjacent guanine rings of the same chain. Furthermore, to accommodate the platinum atom in this structure, the guanine molecules must be tilted away from the DNA helix, and so interfering with replication. Since, the tin complexes were structurally similar to those of platinum; it was expected that their mode of action would also be similar (Crowe *et al* 1984).

Based on these facts we thought it worthy to undertake this investigation on the Genotoxic effect as well as its anti-tumor potentiality of a new organotin compound $Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} in mammalian system. The advantage of this new complex is that it has Sn-N bond lengths of Sn-N (1) = 2.45A° and Sn-N (2) = 2.56A° which is higher than 2.39A°. Another advantage is that this new organotin compound is a diethyltin dihalide adduct. It is therefore believed to be quite unstable and in turn facilitates the formation of a tin-DNA complex.

Formula:

$C_{17}H_{22}N_2Cl_2Sn$;

$Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]}

Properties:

Mol. wt. 444.0; Pale yellow in color. Soluble in ethanol (<2%). Hereafter called OTC

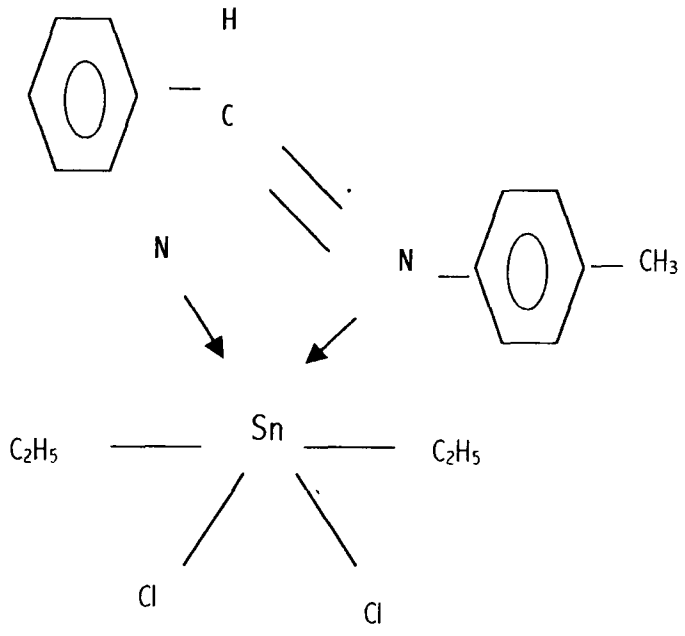
From the background, it is expected this new organotin compound can form better organotin-DNA complex. Therefore the objective of this project is to investigate:

Objective:

1. Genotoxic potentiality of $Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} in both *in vivo* and *in vitro* systems .

2. Establish a relationship between genotoxic effects and the endogenous GSH levels. Since the outcome of the sample exposure to organotin compound is determined by the detoxification status, the level of glutathione-S-transferase and glutathione will be estimated.

3. Anti-tumor activity in the mammalian system.



Structure of OTC

Determination of Lethal dose

Most anticancer drugs have a relatively poor therapeutic index. For this reason the majority of antiproliferative agents in current use are given at very close to the maximum tolerated dose (MTD). This precludes the use of healthy volunteers that makes such studies potentially hazardous. It has been the standard practice to establish the MTD in rodents and extrapolates to man using some form of stepwise escalation to reach a maximum tolerant dose (Phase I), which can be applied in tumor-specific studies of anti-tumor activity (Phase II).

A number of different schemes have been used for determination of a safe starting dose based on animal toxicology. The NCI, US reviewed the data from mice and dogs and concluded that one-tenth the LD_{10} (Lethal $dose_{10}$ – This is the dose of drug which will kill 10% of the exposed animals within 30 days) in mice is a safe starting dose provided this is tolerated in dogs in Phase 1 clinical trial (Judson 1995).

In this study, a few preliminary experiments were carried out so as to determine an appropriate dose for use in all further studies. Some experiments were also carried out with buthionine sulfoximine (BSO; Glutathione depleting agent). (The importance of BSO will be discussed in the following chapter).

Table 1. To determine the $LD_{10/30}$ for a colony of mice exposed to OTC.

Dose	No. of animals exposed	No. of dead animals(%)
0	10	0
5	20	0
15	20	3(15)
30	20	8(40)
BSO+15	20	10(50)
BSO+30	20	13(65)

Therefore, in all further experiments, 15mg kg^{-1} was used, as it appears to be close to the $LD_{10/30}$. Although, in some experiments 30 mg kg^{-1} was also used.

Chapter 1.

Level of Glutathione and Activity of Glutathione-S-transferases

Literature Review

Glutathione (GSH), a tripeptide non-protein thiol present virtually in all animal cells, is synthesized within many cells from its constituent amino acids - cysteine, glutamate and glycine. Linked together in this form, these amino acids provide the cell with an essential compound that performs a variety of critical functions as part of the body's natural defense system. GSH is present in millimolar concentrations and is found throughout the cell, with the bulk in the cytoplasm, subcellular particles, such as the nucleus and mitochondria, having smaller amounts (Biaglow and Tuttle 1993). GSH plays a role in the detoxification and repair of cell injury induced by a wide range of toxic agents, which include cytotoxic drugs, radiation and hyperthermia (Biaglow and Tuttle 1993). It is a major component of several intracellular anti-free radical enzymes, glutathione peroxidase and glutathione reductase. Hence, it protects the cells against damage from free radical. The reactivation of these anti-oxidants requires adequate amount of reduced glutathione.

GSH is probably the most important cellular anti-oxidant. Interestingly, Fahey and Sundquist (1991) found strong evidence for an evolutionary link between glutathione and aerobic eukaryotic metabolism; these findings indicate that glutathione evolved as a molecule that protects cells against oxygen toxicity. Cells that are deprived of glutathione typically suffer severe oxidative damage associated with mitochondrial degeneration. Metabolic utilization of glutathione follows several pathways including reactions catalyzed by glutathione-S-transferase (mercapturate pathway) (Meister 1994). The evolution of glutathione and many glutathione-dependent enzymes has been driven by the need to survive toxic insults. Molecular and genetics studies unequivocally prove their role across all phylogeny. This includes their ability to protect against the toxic effects of chemicals in mammals and man. The relative level of these enzymes in normal and tumor

cells and the ability to manipulate their functions in a tumor-specific manner provides exciting opportunities for chemotherapy (McLellan *et al* 1999).

Glutathione is also synthesized by tumors, some of which (notably drug- and radiation - resistant tumors) exhibit high cellular levels of glutathione and high capacity for its synthesis. Such tumors have a greater requirement for glutathione than do many normal tissues and thus show resistance against radio- and chemotherapy (Meister *et al* 1983; Dethmers *et al* 1981).

Experimental production of glutathione deficiency has been attempted by administration of compounds that react with glutathione. Thus far, the most useful approach to the production of glutathione deficiency has been treatment with specific inhibitors γ-glutamylcysteine synthetase, the enzyme that catalyzed the first and rate-limiting step of glutathione synthesis. One of the inhibitor designed was buthionine sulfoximine (BSO), which inhibits γ-glutamylcysteine synthetase very effectively without any side effects (Meister 1994). Glutathione deficiency sensitizes cells to the effect of radiation, oxidative reactions and to various toxic compounds. These effects have been applied in chemotherapy and radiation systems (Meister 1991). Glutathione deficiency also leads to oxidative stress in many tissues. Mitochondrial and associated cell damage is found in mice treated with BSO. Mitochondria do not synthesize glutathione but obtain it by transport from the cytosol. Several tissues of adult mice are affected by administration of BSO and in young mice more extensive damage is found and there is early mortality due to multi-organ failure (Martensson *et al* 1991). Treatment of peripheral blood mononuclear cells with BSO was found to markedly inhibit their proliferation (Meister 1991), and later work has confirmed that glutathione deficiency decreases lectin-induced proliferation of lymphocytes (Suthanthiran *et al* 1990). Although glutathione is required for proliferation, the mechanism of its function in this system is still unsettled (Mazia 1961; Holmgren 1979).

After the development of amino acid sulfoximine inhibitors of γ-glutamylcysteine synthetase, it was suggested that treatment with these

agents might make tumor cells more sensitive to chemotherapy and to radiation therapy (Meister 1979). The potential usefulness of BSO in the sensitization of cells to radiation was first directly shown in studies on several human lymphoid cell lines (Meister 1983). It was found that treatment of resistant leukemias with BSO led to sensitization of tumors to phenylalanine mustard (Suzukake 1983). In studies with mice bearing such resistant tumors, intraperitoneal infusion of BSO led to sensitization of the tumors and to increase life span of treated animals. These important observations open a way for the clinical trial of BSO, which is now in progress (Ozols *et al* 1988; Hamilton *et al* 1990). Depletion of glutathione by treatment with BSO sensitizes cells to the toxic effect of heavy metals (Singhal *et al* 1987), Cisplatin (Anderson 1990), Cyclophosphamide (Ishikawa *et al* 1990), radiation (Chattopadhyay *et al* 1999), mitomycin C (Dev-Giri and Chatterjee 1998), but with regard to radiomimitic drug bleomycin the presence of BSO reduced the BLM-induced DNA-damage (Chattopadhyay *et al* 1997). Treatment of BSO produces a rapid decrease in the GSH levels of various tissues (Griffith and Meister 1979). Following a single dose of 556mg kg^{-1} BSO, the GSH content of various normal tissues were depleted in a time-dependent manner. Kidney and liver showed the most rapid depletion, intermediate rate of depletion were seen in the lungs and bone marrow of mice with a nadir at 8-12 hours in the later (Lee *et al* 1987). The recovery rates of GSH in the various tissues following a single dose of BSO also differed considerably. Recovery, to the pretreatment values, was most rapid for the liver with 16 hours and comparatively slow in bone marrow with 72 hours. Furthermore, a pronounced 'overshoot' in GSH levels occurred during recovery in various tissues particularly the liver and kidneys (Lee *et al* 1987). Earlier studies have also shown that 10 hours of 200 mg kg^{-1} BSO treatment in mouse bone marrow cells depletes the concentration of GSH to 54% of the control value (Chattopadhyay *et al* 1999).

The effectiveness of many clinically useful anticancer drugs can be severely limited by the development of drug resistance. These include

alteration of drug uptake or drug efflux from the cell, changes in drug-metabolizing enzymes, and changes in target enzymes or DNA repair enzymes (Mattern *et al* 1993). An increased conjugation with glutathione has been proposed as a major mechanism in development of drug resistance towards alkylating agents. This has been attributed to the ability of GSH to compete with DNA for drug binding (Waxman 1993; Tsuchida 1992).

Elevated cellular GSH content has been reported as a mechanism of cisplatin resistance (Andrew 1986; Meijer 1992). Various studies have indicated that glutathione conjugate reactions may be involved in the detoxification of cisplatin. It was also demonstrated that GS-Pt inhibited protein synthesis and could contribute to the toxicity of cisplatin. Of interest, they also provided direct evidence for the ATP-dependent transport of GS-Pt across plasma membrane of L1210 cells. Thus, the elimination of the GS-Pt conjugate from tumor cells may also be an important mechanism that reduces the intracellular platinum accumulation. Richon (1987) had suggested that both decreased cellular level of the drug and increased cellular GSH level are important in the development of resistance to cisplatin.

To assess the contribution of glutathione in this investigation, we felt that it is necessary to determine the level of glutathione in both normal as well as the tumor systems.

Glutathione-S-Transferases (GSTs) belongs to a family of enzymes that catalyze the conjugation of glutathione to a wide variety of chemical toxins (Hayes 1999) and reactive electrophiles (Meister 1988; Moscow 1989). Blockage of the GSH / GST detoxification system enhances the chemosensitivity of several tumors and tumor cell lines. (Armstrong *et al* 1992; Nakanishi *et al* 1997) GSTs are often over expressed in drug-resistant cell lines. There has been little recent progress in clearly establishing the role of these enzymes in the detoxification of currently used anti cancer drugs, although there is some evidence that their polymorphic expression may be a factor in the outcome of chemotherapy. There are several reports of accelerated GSH anti-cancer drug conjugation mediated by the GST (Dirven

et al 1994; Hayes *et al* 1995; Cnubben *et al* 1998). An increased concentration of GSTs is linked to an enhanced detoxification of alkylating agents and therefore, to the development of drug resistance. For several alkylating agents, like melphalan, chlorambucil, and others, the role of GSTs in the formation of glutathione conjugates has been demonstrated. (Ciaccio *et al* 1991; Meyer *et al* 1992; Bolton *et al* 1991; Dirven *et al* 1994.) A concentration of 30 - 240 μM GSTs has been reported in tumors or tumor cell lines (Peters 1994; van der Zee 1992). The GSH conjugation reaction by GSTs is a mechanism by which anticancer drugs can be inactivated intracellularly. Many anticancer drugs were designed to bind target protein or DNA and their conjugation by GSH may interfere with this binding. As there is a high concentration of GSH in most mammalian cells (Meister and Anderson 1983) the competition of GSH with target protein / DNA for drug binding is very significant.

Exposure of mammalian cells to a variety of chemical agents results in increased levels of xenobiotic-metabolizing enzymes, GSTs glucuronosyl transferases, and NAD(P)H: quinone reductase. These enzymes function as intracellular detoxification systems of mutagens, carcinogens, and other toxic compounds (Prochaska *et al* 1985; Talalay *et al* 1987) and, by decreasing the levels of compounds capable of generating reactive oxygen species; they are part of the enzymatic antioxidant defense against oxidative stress (Sies 1991). However, from an analysis of the chemical structures of many compounds, which are GSTs substrates, the electrophilic nature of their cytotoxic moieties is noted to be a common characteristic. These drugs can interact with thiols of reduced GSH, forming a thioether, which is less toxic, and water-soluble (Colvin *et al* 1993). GSTs catalyzed the nucleophilic addition of GSH to electrophilic centres of a wide variety of compounds. This reaction is the first step in the formation of mercapturic acids, a pathway mostly resulting in the elimination of potentially toxic compounds (Boyer 1985; Mannervik 1985). GSTs are also involved in the metabolism of several types of anti-cancer drugs and are over expressed in many human refractory

tumors (Tsuchida 1992). In cell lines resistant to anti-cancer drugs as diverse as alkylating agents, cisplatin etc. increased GSTs and GSH concentrations have been implicated as resistance mechanisms (Tsuchida 1992). High GSH and / or GST levels in tumors may therefore be a barrier to an effective treatment with chemotherapeutics.

The intracellular glutathione conjugates maybe harmful to the cells, as some glutathione conjugates have been found to inhibit GST and glutathione reductase (GR) activity, inhibition of GR may alter the ratio of GSH / glutathione disulfide (GSSG); this will in-turn affect the glutathione-mediated detoxification system (Commandeur 1975). Therefore, the glutathione conjugates must efficiently be disposed from the cells. ATP-dependent transport of glutathione-S-conjugates export pump (GS-X pump) is the most important transporter of glutathione conjugates (Ishikawa 1992).

As mentioned earlier, glutathione-S-transferases catalyze the conjugation of glutathione to a wide variety of chemical toxins (Hayes 1999). Therefore, it would be logical and interesting to determine the rate of activity of the enzyme in both normal and tumor systems, after treatment with OTC, besides determining the level of GSH itself.

Materials and Methods

Measurement of Total Glutathione

In this study GSH was estimated in normal as well as Dalton's lymphoma cells.

In normal cells, mouse bone marrow cells and human peripheral blood lymphocytes were used.

The method of Akerboom and Seis (1981) was followed.

Materials:

A. *Mouse bone marrow cells (BMCs)*

Inbred Swiss albino mice of 6-8 weeks, weighing about 20 - 25gms mostly males were used for all *in vivo* experiments (2n=40, all acrocentric). Mice were maintained in communal cages, under controlled temperature (20°C ± 2) and lighting condition (12h light and 12h dark). Standard animal feed (NMC Oil Mills Ltd., Pune, India) and water was provided *ad libitum*. The bone marrow cells from the femur were isolated just before used.

B. *Human peripheral blood lymphocytes (HPBLs)*

For *in vitro* experiments human peripheral blood lymphocytes (HPBLs) were used. Blood was collected from healthy young donors (age 23 - 35), mostly males. For GSH measurement first of all, lymphocytes have to be separated out from heparinized whole blood on a ficoll-hypaque (FH) density gradient by the method of Boyum (1968).

C. *Dalton's Lymphoma Cells. (DL cells)*

The tumor Dalton's lymphoma was originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, US in 1947.

Subsequently, an ascites form was developed by repeated intraperitoneal transplantation of tumor (Chakrabarti 1984)

Dalton's lymphoma cells was transplanted into the peritoneal cavity of inbred Swiss albino mice, and the tumor cells were taken out 6-7 days after transplantation with the help of a syringe and a 26 gauge needle and used immediately for GSH estimation.

Principle:

The recycling assay or kinetic assay utilizes the continuous glutathione reductase (GR) catalyzes reduction of the sulphydryl reagent 5,5' DTNB (Ellman's reagent) to the chromophoric product 5-thio-2-nitro benzoic acid (TNB) and glutathione disulfide (GSSG). GSSG is reduced to GSH by the action of GR and nicotinamide adenine dinucleotide phosphate reduced sodium salt (NADPH).

The chromophore is monitored spectrophotometrically at 412nm and is proportional to the sum of GSH and GSSG present.

$$2GSH + DTNB \rightarrow GSSG + TNB$$

$$GSSG + NADPH + H^+ \rightarrow GR \rightarrow 2GSH + NADP^+$$

Quantitation is achieved by comparison with a standard curve of known GSH concentration. This procedure is highly specific as it utilizes enzymatically-catalyzed conversion of GSSG to GSH.

Reagents:

$Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} (OTC)

This is synthetic compound synthesized at the Chemistry laboratory, RSIC, NEHU, Shillong. A typical procedure for synthesis is followed. Briefly a stirred solution of aniline in dichloromethane was treated with a solution of Et_2SnCl_2 in dichloromethane. The stirring was continued and the resulting suspension was treated with a solution of N-(2-pyridylmethylene) methylbenzenamine. A

microcrystalline solid obtained was filtered, washed with petroleum ether, recrystallized from the same solvent and dried *in vacuo* (Basu Baul *et al* 1998).

A working solution of 1mg ml^{-1} was freshly prepared in 2% ethanol. For the different doses, (*in vivo* system) the desired concentration was directly injected intraperitoneally in mice from the working solution. For *in vitro* system, a working solution of 10μg ml^{-1} was prepared just before used and different doses were added to the blood.

DL-Buthionine-(S,R)-Sulfoximine (BSO,Sigma,USA).

This is a potent inhibitor of the enzyme γ-glutamyl cysteine synthetase of GSH synthesis pathway. .BSO was freshly prepared in double distilled water. For *in vivo* experiments BSO was injected intraperitoneally. For *in vitro,* BSO was added directly to freshly collected heparinized blood.

Ficoll-hypaque (Sigma, USA).

5 - Sulfosalicyclic acid (5-SSA) (Merck India LTD).

dehydrate 10% and 20%

EDTA - Phosphate buffer 0.1M pH 7.

Potassium dihydrogen 0.1M (solution A)

Dipotassium hydrogen 0.1 M (solution B)

39 ml of solution A was mixed with 61 ml of Solution B and pH adjusted to 7. EDTA was added to achieve a final concentration of 1mM.

5,5' Dithio-bis-2-nitrobenzoic acid (DTNB; Roche Molecular Biochemicals, Germany),

1.5mg ml^{-1} DTNB was dissolved in 0.5% freshly prepared $NaHCO_3$.

Nicotinamide Adenine Dinucleotide phosphate tetra sodium salt (NADPH; Roche Molecular Biochemicals, Germany).
4.0 mg ml^{-1} NADPH was dissolved in 0.5% freshly prepared $NaHCO_3$.

Glutathione reductase (GR; Roche Molecular Biochemicals, Germany).
6 units ml^{-1} was made in 0.1M phosphate buffer, just before used and kept in ice. Freezing was avoided as it leads to denaturation and loss of enzyme activity.

Treatment:

BSO

BSO (50mg kg^{-1}) was injected intraperitoneally 4 and 24 hours before estimation of GSH from DL cells and BMCs *in vivo*.
For *in vitro* system, BSO was administered at a dose of 5mM in the blood at least 3 hours before estimation.

OTC

15 mg kg^{-1} of OTC was used for *in vivo* studies; Estimation was carried out 4 and 24 hours after treatment. *in vitro*, 3μg ml^{-1} of OTC was used and estimation was done after 3 hours.

Sample processing:

1. 50 μl of undiluted sample (BMCs/ HPBLs/ DL cells) suspension was transferred into a micro centrifuge tube containing 150 μl of 10 mM HCl.
   Acid treatment reduces oxidation of GSH to GSSG and to mixed disulfides also inactivates γ- glutamyl transpeptidase, which catalyzes the following reactions that decrease the levels of both GSH and GSSG.

GSH + aa $\Leftrightarrow$ γ Glu - aa + CySH- Gly (Transpeptidation)

GSH + H_20 $\rightarrow$ Glu + CySH- Gly (Hydrolysis)

GSH + GSH $\Leftrightarrow$ γGlu-GSH + CySH- Gly (Autotranspeptidation)

2. Cells were lysed by alternate freezing and thawing at least three times at 10^0C and at room temperature for 10 minutes respectively and centrifuged in a microfuge at 10,000 rpm for 5 min.

3. The supernatant was deproteinised using 100µl of ice-cold 10% 5-SSA with intermittent shaking.

4. The tubes were kept in ice for 10-15 min and the acid precipitable proteins were removed by centrifuging at10,000 rpm at 4^0C for 15 min in a Beckman J2- HS centrifuge. The supernatant was immediately used for GSH determination.

Advantages of the processing method:

1. Acid precipitation of samples maintains proper thiol- disulfide redox status (GSH oxidizes rapidly at pH values above 7.0). Acidification allows for the precipitation of proteins and ensures cell lysis and subsequent release of free thiols and disulfides. It also avoids GGT catalyzed degradation of GSH.

2. 5-SSA was preferable for deproteinization because TCA, perchloric and meta-phosphoric acids do not maintain true GSH: GSSG ratios and also these acids interfere with subsequent enzymatic reactions.

3. Chelating agents such as EDTA were used in the sample buffer so as to prevent iron-mediated formation of peroxides in the presence of oxygen.

Method:

1. To 1ml of buffer taken in a cuvette 50μl of sample suspension, 50μl of NADPH, 20μl of DTNB and 20μl of GR were added. GR was added to initiate the assay.

2. The contents were mixed and the formation of TNB was followed continuously with a record for a total of 5 min at 412nm in a Beckman DU - 640 Spectrophotometer.

3. The amount of GSH was calculated from a standard curve where the GSH equivalents were plotted against the rate of change of absorbency at 412nM.

Standard curve was prepared from a stock solution of 10mM GSH (30.7mg $10ml^{-1}$ in 5% 5 - SSA diluted to 1-10 μmol GSH ml^{-1}. A sample blank lacking GSH was used to determine the background rate.
The values were reported in GSH equivalents as μmol / $1x10^6$ cells in all cases.

Determination of Glutathione - S - Transferase Activity

In this study also the materials and sample processing methods and treatment were same as that of Glutathione estimation describe earlier. The method of Habig et al (1974) was followed.

Principle:

Glutathione - S - transferase (GSTs) was assayed by measuring the rate of enzyme-catalyzed conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene at a final concentration of 1mmol/l GSH, 1mmol/l CDNB and 100 mmol/l potassium phosphate buffer at pH 6.5. A unit of the enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μmol of S-2, 4, dinitrophenyl glutathione per minute using 1mmol GSH and CDNB.

The method estimates all the isoforms of GST (i.e.α, μ, π, ε and θ) using CDNB as a general substrate, which is conjugated to GSH. The reaction depends upon a direct change in the absorbance of the substrate at 340 nm when it is conjugated with GSH. Since the reaction was catalyzed at a finite rate in the absence of the enzyme, care was taken to keep the substrate concentration constant. This reduces the non-enzymatic catalysis. For the substrate, the change in absorbance was a linear function of enzyme concentration and of time for at least 3 minutes.

Reagents:

1. Phosphate buffer 0.1M pH 6.5
   Potassium dihydrogen 0.1M (solution A)
   Dipotassium hydrogen 0.1M (solution B)
   68ml of solution A was mixed with 32ml of solution B and the pH adjusted to 6.5

2. 30mM 1-chloro-2, 4-dinitrobenzene (CDNB; SRL, India)
6.08mg of CDNB were dissolved in 1ml of 95% ethanol. (CDNB is not soluble in water).

3. 30mM reduced glutathione (GSH; Sigma, USA) 9.21mg of GSH were dissolved in 1ml ddw.

(Reagents 2 and 3 were prepared fresh)

Sample Processing:

The materials were processed in a similar way described earlier in the GSH estimation section.

Method:

The following solution was taken in a glass cuvette in the given proportion.

1 ml phosphate buffer
0.1 ml CDNB
0.1 ml of the sample (enzyme source)

The volume is adjusted to 2.9 ml with distilled water, and the reaction mixture is preincubated in the Beckman DU - 640 Spectrophotometer at 37°C for 5 minutes.

The reaction is initiated by adding 0.1 ml 30mM GSH. The absorbency is taken at 340 nm for 5 minutes. Reaction mixture without the enzyme is used as the blank.

Calculation - μmol of CDNB-GSH conjugate formed/min/mg proteins

$$\frac{OD \times 3 \times 1000}{9.6 \times 5 \times \text{protein in mg}}$$

9.6 is the difference in the millimolar extinction co-efficient between CDNB-GSH conjugate and CDNB.

Protein Estimation

Principle:

Folin-Ciocalteau reaction is based on the color production by two reactions: Lowry's method for protein estimation is followed.

Biuret reaction of proteins where -CO-NH- group present in the proteins chelates copper ions in alkaline solution and reduction of the phosphomolybdic-phosphotungstic acid reagent to molybdenum by the tyrosine and tryptophan residues presents in the proteins.

Reagents:

1. Alkaline copper sulfates ($CuSO_4$) solution. (Merck India LTD).

   (i) 1% $CuSO_4$ solution
   (ii) 2% Sodium – potassium tartarate (Na-K tartarate).
   (iii) 2% Sodium Carbonate (Na_2Co_3) solution in 0.1N NaOH.

The solution was prepared just before use by mixing solution solutions (i), (ii) and (iii) in the proportion (1:1:100)

2. 1N Folin-Ciocalteau phenol reagent (SRL Pvt. Ltd. India)
   The commercially available 2N Folin Ciocalteau

phenol reagent was diluted in double distilled water (1:1)

3. Bovine serum albumin (BSA; Sigma, USA) A standard curve was prepared using BSA in the range 5 - 75μg ml^{-1}.

Method:

3 ml of alkaline $CuSO_4$ solution was taken in a test tube. 100 μl of the diluted sample was added to it and the tubes were kept at room temperature for 10 minutes.

Then 300μl of Folin-Ciocalteau phenol reagent was added to the tubes and incubated in the dark for 30 minutes.

The optical density was measured at 750nm in a Beckman DU - 640 Spectrophotometer against a reagent blank containing all the solutions except the sample.

Protein content of the sample was calculated from the standard curve prepared using BSA as a standard.

Student's t-test was performed in order to see the significance of the difference between the observed values of all the treated and untreated samples.

Results

The data of endogenous GSH level and GST activity in all treated and untreated samples are presented in tabular form.

Total Glutathione level

In mouse bone marrow cells, the glutathione content at normal condition was 4.67 μmol/1X10^6 cells, which drops to 2.88 μmol/1X10^6 cells after 24 hours of BSO treatment. The drop in GSH level is about 42%. An interesting observation made, was that the level of GSH increased notably 4 hours after OTC administration raising the level to 10.78 μmol/1X10^6 cells. There is an overall increase of about 55% in GSH level (Table 1.1).

A similar trend was also observed in human peripheral blood lymphocytes *in vitro*. The level of GSH dropped by 85% after 3 hours BSO treatment, from 5.60 μmol/1X10^6 cells to 0.84 μmol/1X10^6 cells. The level of GSH increased considerably after single OTC administration. An increase of 23% from 5.60 to 6.88 μmol/1X10^6 cells was observed (Table1.2).

In DL cells the rate of depletion was more after 4 h than 24h of BSO treatment. The depletion of GSH was 75% after 4h and 47% after 24h. 4h of OTC treatment elevates the GSH level by 79% compared to the untreated animals (Table1.3).

Glutathione -S- transferase activity

The rate of activity of the enzyme glutathione-S-transferase (GSTs) is another aspect of the work carried out in this study. When exposed to toxic insults, in this case the drug OTC, the rate of the enzyme activity increased notably in both normal and tumor systems. In mouse bone marrow cells, the rate of activity of the enzyme was 21.55 μmol/min/mg proteins in untreated conditions and the activity was raised up to 40.33 μmol/min/mg proteins and

25.14 μmol/min/mg protein after 4 hours and 24 hours treatment respectively (Table 1.4).

In human peripheral blood lymphocytes, the rate of GST activity went up to 26.70 μmol/min/mg proteins from 16.19 μmol/min/mg proteins after 3 hours of the drug treatment (Table 1.5).

Similar observations were also made in case of DL cells. The rate of GST activity in untreated conditions was 27.86 μmol/min/mg protein which up to 50.44 μmol/min/mg protein, after 3 hours of the drug administration. There was no increase of activity of the enzyme after 24 hours treatment, where the enzyme activity is as good as that of the control, i.e. 25.53 μmol/min/mg proteins (Table 1.6).

Discussion

Glutathione and glutathione-dependent enzymes have been known to be of central importance in the detoxification of peroxides, hydroperoxides, xenobiotics and drugs (Chasseaud 1979). Reduced glutathione levels in the tumoral tissues were found to be higher than those in the normal tissues (Meister 1988). GSH contents have been found to be elevated in cancer tissue and cell lines (Murray 1987, Mickisch 1991, Blair 1997). Present data shows that the level of GSH was higher in DL cells than in BMCs. Increased tumoral tissue GSH levels may be a consequence of the increased detoxification activity in the tumor cells. Tumoral GSH levels reported in the literature show considerable differences, probably due to the heterogeneous tissue structure, the storage conditions of the samples or the methods used in analysis (Meister 1988; Taniguchi *et al* 1989). At this point, it should be stressed that the samples used in our study were always freshly taken from the animals.

In mouse BMCs, 42% depletion was observed after 24h of BSO treatment. It has been reported that following a single dose of 556 mg kg^{-1} BSO treatment, the GSH concentration in various tissues were depleted in a time dependent manner. Intermediate rate of depletion was seen in bone marrow cells with a nadir at 8-12h (Lee *et al* 1987). The observed GSH content of BMCs following depletion was 17% of the initial level. Therefore the incubation period for BSO-treatment was kept at 10h in the present study. However in an earlier study, 54% of endogenous GSH-depletion was achieved after 10h of BSO-treatment at 200mg kg^{-1} (Chattopadhyay 1999). Thus, in the present study we did not perform the endogenous GSH level measured after 10h of BSO-treatment.

In case of in vitro study the freshly drawn blood was incubated with BSO for 3h before addition of OTC for another 2 more hours. It has been observed that in cultured cells >75% depletion of GSH was achieved within 4-5h by 500μM – 10mM of BSO (Shrieve *et al* 1985; Edgren *et al* 1987). In

this study, the concentration of BSO used was 5mM since significant sensitization by BSO was observed at this concentration with respect to radiation- and bleomycin induced chromosomal aberrations (Chattopadhyay 1998; Chattopadhyay 1997).

For DL cells, since they are growing aseptically in the mouse peritoneal cavity, 50mg kg^{-1} of BSO was used and after incubation for 4 and 24h, 75% and 47% depletion of endogenous GSH-level was achieved respectively. In this study an interesting observation was made regarding the elevation of endogenous GSH level in all the samples following OTC-treated alone. Exposure of human ovarian cancer cell lines to cisplatin induced a marked increase of GSH-synthesis associated with resistance to the treatment. (Godwin *et al* 1992) and such increase was also reported in glial cells 24h after triethyltin and trimethyltin treatment (Cookson 1998). The observed increase in GSH level might be one of the mechanism(s) for detoxification, which may be responsible for drug resistance.

The cisplatin inactivation through the formation of GSH-Pt conjugation was observed earlier (De Graeff *et al* 1988) and if this is true then GSH-S-transferase (GSTs) must be involved catalyzing the glutathione conjugate formation (Hall *et al* 1994; Bolton *et al* 1991). Therefore, it is worth measuring GSTs activity in the present study after observation of elevation of endogenous GSH level by OTC treatment. There is an increased of around 45% of GSTs activity in all the systems. Recent findings show that GSTs have an important role in carcinogenicity and resistance to drugs, and is elevated in several tumors (Anttila et *al* 1995; Jensson *et al* 1985; Niitsu *et al* 1989). GSTs are enzymes that catalyzed the conjugation of cisplatin to GSH. The cisplatin – GSH complex has been proposed to be ejected from the cell in an ATP-dependent fashion by the glutathione S-conjugate (GS-X) export pump (Ishikawa and Ali-Osman 1993; Goto *et al* 1995). ATP-dependent transport systems, referred to as pumps, are proposed to be responsible for resistance to multiple drugs i.e. multidrug resistance (MDR) (Biedler 1992). GSTs are directly implicated in the protection of cells against many cytotoxic

and carcinogenic chemicals (Doroshow *et al* 1990; Raunio *et al,* 1995). Although there are evidences from studies of both cell-lines and clinical materials that increased expression of GSTs and raised level of GSH maybe associated with resistance to cytotoxic drugs, the relationship between response and expression in patients remain unclear (Ghazal-Aswad 1996).

Conflicting data have been reported about the association of GSTs in detoxification of cytotoxic drugs in human ovarian *in-vitro* models (Lewis *et al* 1988; Mistry *et al* 1991, Saburi *et al* 1989; Batisy *et al* 1986). Moreover, the relationship between GST expression / activity and resistance to cisplatin-based treatment in primary ovarian cancer patients has yet to be clarified (Ferrandina 1997). The role of GSTs in predicting clinical outcome in ovarian cancer patients has been extensively investigated. Some authors even using different technique to assess both GST isoenzyme pattern and total GST activity (van der Zee 1995; Wrigley 1996) failed to find any relationship between GSTs avtivity and time of progression and overall survival (Ferrandina *et al* 1997), analyzed the clinical role of GST activity levels in a large series of primary untreated ovarian cancer patients who underwent surgery. Unexpectedly, high GST activity levels were demonstrated to be associated with better chance of response to chemotherapy and a more favorable clinical outcome. Codegoni (1997) also reported similar observation where high GSTπ mRNA levels in ovarian cancer biopsies are directly associated with a better survival. In this study, our observation is in agreement with the data reported by Ferrandina and Codegoni showing higher GST level / activity in tumor samples taken after treatment, and can directly be implicated to progression free and better survival rate of the animals.

A tentative explanation of these findings is that high intra-cellular production of toxic oxygen metabolites reflecting some metabolic dysfunction of the tumor cells could also induced GST expression. In this context, high GST levels may simply reflect a situation of abnormal oxidative metabolism of tumor cells, which could become more vulnerable to the additional stress

of the drug treatment. GSTs are only part of a complex detoxification system, since multiple enzymes are involved in the redox cycling of glutathione (Larsson 1983) and therefore, insight into the kinetics of glutathione and glutathione-dependent enzymes could yield a more dynamic and reliable representation of the function of this system. Chemotherapy induced modification of GST levels / activity in samples taken before and after treatment might also provide useful information about the biology of the system (Ferrandina 1997).

Of all the mechanisms implicated in preclinical studies GSH and its metabolism is the most commonly identified in resistance to cisplatin and carboplatin. GSH functions primarily through nucleoplilic thioether formation and peroxidation/reduction reactions. The exact mechanism by which GSH decreases the toxicity of cytotoxic drugs like cisplatin is not well established; but the following possibilities have been proposed (De Graeff *et al* 1988) alteration of platinum complex transport, drug inactivation through the formation of an inactive GSH-Pt, decreased binding of platinum to DNA and increased DNA-repair. More recently data have been published which indicate that cisplatin maybe extruded from cells as a glutathione conjugate, and that increased expressions of the GS-X pump maybe associated with drug resistance (Ishikawa and Ali-Osman 1993; Ishikawa *et al* 1994).

The data obtained in this section forms the base line on which the rest of this investigation depends.

Table 1.1. Level of Glutathione in mouse bone marrow cells after treatment with BSO (50 mg kg^{-1}) and OTC (15 mg kg^{-1})

Sample #	BSO treatment (h)	OTC treatment(h)	Total GSH μmol 10^{-6}	Mean ± SEM (increment %)
1.	Untreated	-	05.70	
2.	,,	-	03.00	
3.	,,	-	04.80	
4.	,,	-	05.80	04.7 ± 0.64
5.	24	-	02.90	
6.	,,	-	02.70	
7.	,,	-	03.00	
8.	,,	-	02.90	02.8* ±0.06
9.	-	4	09.80	(-42%)
10.	-	,,	10.70	
11.	-	,,	10.60	
12.	-	,,	12.00	10.8# ± 0.45 (55%)

* $p < 0.05$; # $p < 0.01$; Students' t – test compared to the control value.

Table 1.2. Level of Glutathione in human peripheral blood lymphocytes after treatment with BSO (5mM) and OTC (3μg ml^{-1})

Sample #	BSO treatment(h)	OTC treatment(h)	Total GSH μmol 10^{-6}	Mean ± SEM (increment%)
1.	Untreated	-	6.50	
2.	,,	-	3.70	
3.	,,	-	6.50	
4.	,,	-	5.60	5.60 ± 0.66
5	3	-	0.85	
6.	,,	-	0.78	
7.	,,	-	0.98	
8.	,,	-	0.79	0.84* ± 0.05
9.	-	3	6.24	(-85%)
10.	-	,,	5.00	
11.	-	,,	6.16	
12	-	,,	8.72	
13.	-	,,	8.28	6.88# ± 0.70 (23%)

* $p < 0.01$; # $p < 0.05$; Students' t – test compared to the control value.

Table 1.3. Level of Glutathione in Dalton's Lymphoma Cells transplanted in mice after treatment with BSO (50 mg kg^{-1}) and OTC (15 mg kg^{-1})

Sample #	BSO treatment (h)	OTC treatment(h)	Total GSH μmol 10^{-6}	Mean ± SEM (increment%)
1.	Untreated	Untreated	12.60	
2.	,,	,,	08.60	
3.	,,	,,	12.30	
4.	,,	,,	13.20	
5.	,,	,,	07.70	
6.	,,	,,	06.50	
7.	,,	,,	06.00	
8.	,,	,,	07.30	
9.	,,	,,	09.40	
10.	,,	,,	06.60	
11.	,,	,,	06.90	
12.	,,	,,	05.80	08.6 ± 0.77
13.	24	-	03.10	
14.	,,	-	03.00	
15.	,,	-	03.90	
16.	,,	-	07.10	
17.	,,	-	05.00	
18.	,,	-	05.20	
19.	,,	-	05.20	04.6* ±0.54
20.	4	-	01.50	(-47%)
21.	,,	-	01.70	
22.	,,	-	03.00	
23.	,,	-	02.40	
24.	,,	-	02.10	02.2# ±0.26
25.	-	4	16.85	(-75%)
26.	-	,,	16.92	
27.	-	,,	16.96	
28.	-	,,	10.85	15.4#± 1.51 (79%)

* $p < 0.01$; # $p < 0.001$; Students' t – test compared to the control value.

Table 1.4. Glutathione-S-Transferase Activity in mouse bone marrow cells after treatment with OTC (15 mg kg^{-1})

Sample #	OTC treatment(h)	Protein level mg 10^{-6} cells	GST- activity µmol GSH-CDNB min/mg protein.	Mean ± SEM
1.	Untreated	0.95	22.60	
2	-	0.80	20.50	
3.	-	1.01	20.00	21.55 ± 0.79
4.	4	0.78	38.64	
5.	-	0.67	42.65	
6.	-	0.64	37.07	
7.	-	0.52	39.29	
8.	-	1.05	34.36	
9.	-	0.95	46.00	
10.	-	0.81	38.88	
11.	-	0.80	52.57	
12.	-	1.03	33.49	40.33* ± 1.99
13.	24	0.46	24.26	
14.	-	0.38	25.57	
15.	-	0.35	25.60	25.14 ± 0.44

* $p < 0.001$ Students' t – test compared to the control value.

Table 1.5. Glutathione-S-Transferase Activity in Human peripheral blood lymphocytes after treatment with OTC (3µg ml^{-1})

Sample #	OTC treatment(h)	Protein level mg 10^{-6} cells	GST- activity µmol GSH-CDNB min/mg protein.	Mean ± SEM
1.	Untreated	0.88	19.46	
2.	-	0.61	12.87	
3.	-	0.60	17.68	
4.	-	0.80	14.75	16.19 ± 1.47
5.	3	0.73	28.28	
6.	-	0.80	26.73	
7.	-	0.85	25.41	
8.	-	0.90	26.95	26.70* ± 0.58

*$p < 0.01$ Students' t – test compared to the control value.

Table 1.6. Glutathione-S-Transferase Activity in Dalton's Lymphoma cells after treatment with OTC (15 mg kg^{-1}).

Sample #	OTC treatment(h)	Protein level mg 10^{-6} cells	GST- activity µmol GSH-CDNB min/mg protein.	Mean ± SEM
1.	Untreated	1.80	25.07	
2.	-	1.90	26.31	
3.	-	1.70	26.70	
4.	-	1.80	26.66	
5.	-	2.00	30.90	
6.	-	1.28	31.53	27.86 ± 1.09
7.	4	1.10	37.83	
8.	-	1.05	37.83	
9.	-	0.82	48.23	
10.	-	1.12	37.55	
11.	-	1.27	57.00	
12.	-	1.01	59.78	
13.	-	0.97	64.05	
14.	-	1.39	56.44	50.44* ± 3.86
15.	24	0.55	26.80	
16.	-	0.83	22.85	
17.	-	0.63	26.93	25.53 ± 1.33

* $p < 0.001$ Students' t – test compared to the control value.

Chapter 2.

Genetic Toxicology of the drug $Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]}.

Literature Review

Most cytogenetic studies today involve the examination of metaphase chromosomes. The study of chromosome damage at metaphase allows for the observation of a greater number of division figures and presents a more precise and detailed picture of the effects of any mutagenic / carcinogenic agent than does anaphase or telophase analysis. The early history of anticancer drugs development paralleled some of the earliest experiments in mutagenesis. Anticancer drug research has attempted to design direct DNA alkylators targeted to specific DNA sequences (Acromone *et al* 1989). Many of these new drugs, are still likely to have mutagenic and / or carcinogenic potential. Conventional approaches to mutagenic testing, such as chromosomal aberration and sister chromatid exchanges are considered routine for alkylating chemicals or those which give recognizable structural alerts (Ashby and Paton 1993). It is therefore, important to assay the potential long-term consequences of human exposures. A full exploitation of these drugs as a resource, to develop an understanding of mechanisms of mutagenesis and / or carcinigenesis goes beyond the notion of an electrophilic species contrary to the earlier belief that "the property which is common to all the diverse types of chemical carcinogen is that they can form directly, or are metabolized to, reactive electrophilic forms" (Miller and Miller 1966.) The mutagenic properties of anticancer drugs vary considerably depending on their structures and mechanisms of cytotoxicity, but pattern can be discerned, according to their mode of action. (Ferguson 1995). Over the last two decades, several specific and general reviews have been published on the mutagenicity of several drugs e.g. bleomycin (Povirk and Austin 1991), cyclophosphamide (Anderson *et al* 1995). Unfortunately, even for the most widely used anticancer drugs, there are gaps in the literature that makes it difficult to assess the relative mutagenic potential of different types of compound (Ferguson 1995).

In this investigation the mutagenicity of this new organotin compound was carried out, since there is a requirement for such testing before new drugs are released (Anon 1989). These regulations generally require that a package of assays be done, using a number of different endpoints, before it can be concluded that a chemical is non-mutagenic. Cytogenetic endpoints in bone marrow and unscheduled DNA synthesis in rodent liver are required for mutagenecity testing; these assays may be supplemented by others, such as sister chromatid exchanges (SCEs) and chromosomal aberration (CAs) (Anon 1989). The phenomenon of sister chromatid exchanges (SCE) and chromosomal aberration have been long considered as cytological indicators of chromosome damage and repair (Latt, 1980). In this present investigation, these two established end-points besides cell cycle kinetics were used.

Cell cycle

Normal cell cycle progression relies on the cell's ability to translate extra-cellular signals such as mitogenic stimuli and intact extracellular matrices in order to efficiently replicate DNA and divide. The morphological changes associated with cell division have been observed repeatedly since the early 1800s, by light microscopy. A rapid organized procession of events in cellular mitosis includes chromosomal alignment in metaphase, sister chromosome segregation in anaphase, and subsequent division of cellular material leading to the next interphase where cells only appear to grow gradually in size. (Nasmyth 1996) It was not until 1951 when Howard and Pelc reported the synthesis of nucleoprotein in bean root cells (Howard and Pelc, 1951) (otherwise known as DNA synthesis or S - phase) during interphase that the cell cycle, as we know it was born. Strict regulation of this cellular division cycle is essential to ensure proper duplication of genetic information with extremely high fidelity as well as to monitor correct segregation of this information during mitosis.

A tumor-suppressor gene, p53, encodes a 53 kDa nuclear phosphoprotein (known as the p53 protein) involved in the control of cell cycle, apoptosis and DNA repair (Vogelstein 1992; Steele 1998) Consequently, loss of normal p53 function leads to genomic instability characterized by an increased frequency of gene amplification (Livingstone1992; Yin 1992), chromosomal rearrangements and mutations (Bouffler 1995; Havre 1995). Therefore, p53 often referred to, as the guardian of the genome is one of the important players in mediating a response to DNA damage. Specific interaction of p53 with DNA up-regulates the expression of p53 target genes like GADD45 and p21 These act co-ordinately to arrest the growth of cells following DNA-damage. The cellular response to genotoxic agents triggers a rapid increase in the total p53 levels mainly through prevention of p53 degradation.

It has been recognized for a long time that radiation exposure to cells delay their entry into mitosis (Giese, 1947) and this is termed as mitotic delay. It is a general observation that the delay depends on the stage of the cell cycle and a relationship exists between radiation dose and the resulting mitotic delay regardless of the cell line used (Collyn-d'Hooghe *et al*, 1981). Radiation induces delay in the cell cycle due to blocking of the cells at the G1/S or G2/M boundaries of the cell cycle (Nagasawa *et al* 1984). Cell cycle studies have gained importance owing to the fact that the most widely used anticancer drugs seems to work by preventing or delaying cell proliferation and DNA synthesis.

Employing the FPG staining technique (Goto *et al* 1975), cell cycle delay can be measured in terms of reduction in the frequency of 2^{nd} and subsequent division metaphases, and corresponding increase in the first cycle metaphases, at a given time following the treatment. In this investigation, we have tried to determine the cell cycle kinetics of mouse bone marrow cells *in vivo* and human peripheral blood lymphocytes *in vitro*. The delay in the cell cycle kinetics was then determined after the drug treatment.

Chromosomal Aberrations (CA)

Chromosome damage constitutes a set of efficient, reliable and economical criteria to measure genetic toxicity. One of the major types of chromosome abnormalities, which cause genetic disturbances of the cell, is chromosome breakage, inducing damage directly in the genetic apparatus. Thus many experiments have been designed to find out details of the mechanism of drug action, relation to the cell cycle, the fate of aberrations, localization of breaks, and other parameters. For drug assay, two main questions are asked: (a) whether an agent can cause chromosomal damage; and (b) if so, how much. If these two questions are answered, some other tests or details can be omitted. Therefore, the methodology and the protocol should be geared towards these two objectives (Hsu, 1982).

Chromosome alterations have been studied for nearly a century, and it was immediately appreciated that they are associated with malignancy (Boveri, 1902). Numerous *in vitro* and *in vivo* studies have shown that cytogenetic alterations can result from exposure to chemicals, and ionizing and non-ionizing radiation. The association between specific cytogenetic alterations and tumorigenesis is strong (Mitelman, 1994). Indeed, it is this relationship that is used as one justification for including endpoints in toxicological evaluation of industrial chemicals, and development of new pharmaceutical and therapeutical compounds.

Sister Chromatid Exchanges (SCE)

SCEs are S-phase phenomena i.e. they occur during DNA-synthesis and SCE-inducing agents are thought to exert their effect at this phase of the cell cycle (Wolff, 1974). SCE formation appears to be a consequence of errors of DNA replication, possibly at the replication fork itself (Painter, 1980). Sister chromatid exchange analysis has come into use as a sensitive means of monitoring DNA damage. It was also suggested that SCE is a

means by which the cell copes with DNA damage (Evans 1977; Shafer 1982). Increased SCE is an indirect measurement of mutation resulting from DNA- damage and of mitotic rearrangement of genetic material. Consequently, SCE analysis must be relevant in the evaluation of genetic hazards (Benditt and Benditt, 1973). SCE method may have its application as a supplementary diagnostic tool in the analysis of breakage syndromes (Ray and German, 1982) and in monitoring chemotherapy (Raposa, 1982) and have been used for many years as a mutagenicity test (Dean and Danford 1984). Although most of the anticancer drugs currently in the clinic are effective in causing chromosomal damage, there is considerable variability in their ability to cause SCE (Ferguson 1995). For many of the alkylating agents including cisplatin, melphalan and mitomycin C, SCE does indeed occur at lower concentrations and appears to be a more sensitive assay than direct measurement of chromosomal aberration (Sieber and Adamson, 1975). The fact that there is no direct association between SCE induction and an adverse cellular or health outcome, their analysis has limited value in risk assessment, except as a biological dosimeter or biomarker of exposure. Nevertheless, any discussion of cytogenetics would be incomplete without at least some mention of this parameter. All SCE-inducing agents that have also been examined for their effects on DNA synthesis inhibit the rate of DNA-replication (Schwartz, 1983).

Tin is an element widely distributed in the mammalian food chain as well as in soils, water and plants (Koch *et al* 1956; Schroeder *et al* 1964). Organotin compounds have varying degrees of toxicological properties, depending on the nature and number of alkyl groups bonded to the tin atom. The toxic action of many organotin compounds has been ascribed to their tendency to combine with coenzymes and enzymes possessing dithiol group. The mammalian toxicity however, decreases with increase in chain length of the alkyl residue and the nature of the alkyl group is of prime importance in determining their toxicity towards particular living species (Singh and Sharma 1992). The di- and tri- methyltin and di- and tri-ethyltin halide exhibit

neurotoxic properties and present serious health hazards to the nervous system (Sherman and Huber 1987). Almost all of the low-molecular weight di- and tri-alkyl compounds possess some anti-proliferative properties and most compounds produce these properties if they are capable of hydrolysis (Sherman and Huber 1987). Our current knowledge on cellular effects of organotins is mainly restricted to studies with cells of the immune system because of the predominant immunotoxic effects of various organotin homologues (Boyer 1990). Besides direct cytotoxicity, di- and tri-substituted organotins affect cell energetics, membrane-associated functions and macro-molecular synthesis in particular protein synthesis (Penninks 1990; Al-Imara 1993) Previous studies have also demonstrated that triphenyl tin acetate and triphenyltin hydroxide reduced thymocytes and lymphocytes viability (Attahiru *et al* 1991; Bollo *et al* 1996; Vos *et al* 1984), thymus and spleen weight (Vos *et al* 1984), induced central nervous system and respiratory depression (Attahiru *et al* 1991) and lung, liver, intestine and kidney histopathologic lesions in sub chronic toxicity study (Attahiru *et al* 1991). Organotins have been known to increase cytosolic Ca^{2+}, alter functionality and induce apoptosis in rat thymocytes (Gennari 1997). *In vitro* exposure diminishes the viability of mouse spleen cells, decrease antibody secreting cell numbers in fish (Al-Imara 1993;Thompson 1996; Rice 1995) and also inhibit DNA synthesis of spleen cells from mice (Al-Imara 1993). But the possible interaction of this organotin compounds with native DNA has still to be investigated. Moreover, the genotoxic potentialities of this new organotin compound in relation to the cellular glutathione (GSH) level were also investigated, since it plays an important role in cellular defense mechanisms (Deleve *et al* 1991). It is known that GSH protects cells against radiation (Chatterjee *et al* 1986) and various toxic effects of xenobiotics (Shaw *et al* 1986), but not against radiomimetic drugs like bleomycin (Chatterjee *et al* 1989,Chattopadhyay *et al* 1997).

Therefore, an assessment of the influence of GSH on the activities of OTC as well as other organotin compounds is warranted, in order to define

better the compounds whose activity maybe potentiated through appropriate modulation of GSH especially those which show anti-tumor activity.

Materials and Methods

Materials:

A. Mouse bone marrow cells (BMCs)
Described earlier in chapter 1.

B. Human peripheral blood lymphocytes (HPBLs)
Described earlier in chapter 1.

Reagents:

$Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} (OTC).
The preparation and concentration of the drug was described in the 1st chapter.

5-bromo-2-deoxyuridine tablet/powder (BUdR).
For *in vivo* experiments, paraffin coated tablet (50mg) (Roche Molecular Biochemicals. Germany) was implanted subcutaneously in each mouse. For *in vitro* experiments BUdR powder (Sigma, USA) was used after dissolving it in RPMI 1640 culture medium.

Phytohemagglutinin M (Gibco, USA).

RPMI - 1640 (Hyclone, USA) with penicillin and streptomycin (Hyclone, USA).
New Born Calf Serum (Hyclone, USA)

DL-Buthionine-(S,R)-Sulfoximine (BSO, Sigma, USA)
The preparation was described earlier.

Reduced Glutathione (GSH; Sigma, USA);
Freshly dissolved in culture medium before used.

Bis-benzimide (Hoechst 33258) (Sigma, USA)
50μg ml^{-1} working solution was made in double distilled water

Giemsa, 3%, (BDH chemicals, Ltd. UK.)

All other chemicals are of analytical grade.

Treatment:

BSO
BSO was injected intraperitoneally at a dose of 200 mg kg^{-1} 10 hours before the OTC treatment in all *in vivo* experiments.
For *in vitro* system, BSO was administered at a dose of 5mM at least 3 hours before OTC treatment.

GSH
GSH was injected to mice at a concentration of 400mg kg^{-1} half an hour before the OTC and in *in-vitro* system GSH was added at a dose of 15mM 30 mins before OTC treatment.

OTC
15 and 30 mg kg^{-1} of OTC was used for *in vivo* studies. The BMCs were fixed at 17, 20 and 24 hours after treatment, and *in vitro*, 1 and 3 μg ml^{-1} blood were used and cells were fixed at 72 hours.

Culture procedure:

Cultures were set up in medium containing RPMI - 1640 with antibiotics and supplemented with 10% heat inactivated newborn calf serum. In order to stimulate the G_0 lymphocytes 0.2 ml of phytohaemagglutinin M was added in each culture. BUdR (6 μg ml^{-1}) of was added to each culture. All cultures were incubated at 37°C. After about every 12h cultures were gently shaken. Cultures were harvested at 72h to analyze the effect of OTC. All experiments were repeated at least 3 times.

Preparation of Metaphases and differential staining:

The animals were sacrificed 17h, 20h, 24h, after OTC treatment by cervical dislocation with 2h prior treatment of colchicine (10 mg kg^{-1}) in each animal. The femur bones were dissected out and the BMCs were obtained by flushing out with 2ml pre-warmed (37°C) 0.075M KCl with the help of a hypodermic syringe and a 26-gauge needle. A single cell suspension was made in hypnotic solution and incubates at 37°C for 15 min. The cells were centrifuged at 1200 rpm for 5 min and fixed in two changes of fixative for 30 and 10 min respectively, re-suspended in 1ml of fixative and dropped onto a grease free chilled slides and flame dried.

In *in-vitro* experiments, colchicine was added at a concentration of 0.01μg ml^{-1} 3h prior to harvesting. Hypotonic treatment was done for 20 minutes and cells were fixed and slides were prepared according to the flame drying method, as stated above.

Differential Staining:

Differential sister chromatid staining was carried out by the method of Goto *et al* (1975). Slides were treated for 10 minutes with Hoechst 33258 at room temperature in the dark. The slides were then rinsed with distilled water,

mounted in 2xSSC and kept in sunlight for 30-40 min depending upon the intensity of light. After rinsing in distilled water, slides were then stained in 3% Giemsa, air-dried and mounted in DPX.

Scoring and statistical analysis:

Slides were coded at random. For scoring cell cycle kinetics, metaphases were categorized as the first, second and subsequent division cycle based on their differential pattern. The cell cycle data were presented as average generation time (AGT) which is a ratio of BUdR duration (H) and replicative index number of cells (RI) where RI=1xM1+2xM2+3xM3/ number of cells.
At least 100 well spread first cycle metaphase plates (with a few exception) were selected for aberration study from each mouse and also from each culture. For BMCs, chromosomal aberrations were classified as chromatid breaks (chd. bks.), and isochromatid breaks (Isochd. bks.). In case of HPBLs, deletions and chromatid breaks were scored. Total numbers of normal as well as aberrant metaphases were scored. Data were subjected to statistical analysis using the 2x2 contingency test for the frequency of aberrant metaphases and simple χ^2-test for aberration. The actual observed data were used during statistical analysis.
SCEs were scored from 2^{nd} cycle metaphases, at least 25 (with a few exceptions) well spread second cycle were considered for SCE scoring. Metaphase cells with differentially stained sister chromatids from each culture were studied for evidence of SCEs. The data of observed SCE-increase per metaphase were obtained after subtracting the control value from each treatment and the expected SCE-increase per metaphase were presented after adding the individual observed value. Data were subjected to parametric statistical analysis. To compare the effects of OTC with GSH or BSO on the intracellular distribution of SCEs within individual group, the dispersion coefficient H (Snedecor and Cochran 1967) that is the ratio of the sample variance to the sample mean (Margolin *et al.* 1968) was analyzed.

p53 immunoblotting analysis

Reagents:

RIPA buffer (Radio Immuno Precipitation Assay)

Composition of RIPA buffer.
1%(w/w) Nonidet P-40(NP-40)
1%(w/v) sodium deoxycholate)
0.1% (w/v) SDS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2
2mM EDTA
50mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 stock solution.(can be stored in plastic container at room temperature.
100U/ml aprotinin (trasylol, Pentex/Miles).

DNAse (Sigma, USA).

Genei protein estimation kit by bicinchonimic acid (BCA) method. (Bangalore Genei Pvt. Ltd.)

SDS-polyacrylamide gel kit (Bangalore Genei Pvt. Ltd.).

Method:

The level p53 protein in OTC-treated mouse BMCs was evaluated by Western Blotting analysis previously described by Perego (1997). The mouse BMCs was collected 6h after OTC treatment.

The BMCs (untreated and treated samples) were washed in PBS. 200µl RIPA buffer (Radio Immuno Precipitation Assay) was added to the samples in ice. To this, DNAse(1U ml^{-1}) was added, mixed well and protein assay was done base on the bicinchonimic Acid (BCA) method, and using the Genei's BCA Protein Assay kit (KT-31).

To prepare BCA working Reagent (BWR), 50 parts of Reagent A was mixed with 1 part of Reagent B. 2ml of this reagent was added to each sample, mixed well and the optical density was measured at 562nM after incubation for 30 mins at $37^{O}C$. Using a standard curve, the protein concentration of the samples was determined.

40µg of protein was resolved by SDS-PAGE on a 10% polyacrylamide resolving gel and a 5% stacking gel at 50 V constant voltage for 2 h. The proteins were transferred onto a 0.45-µm nitrocellulose membrane at 50 V constant voltages for 7h.

Membranes were blocked at room temperature in TBST containing 5% (w/v) nonfat milk. The membrane was suspended in primary antibody p53 Ab-8(DO-7+bp53-12) (Neomarkers, USA) along with assay buffer for 30mins and then washed in Washing buffer three times for at least 10 mins each time.

The blot was then immersed in second antibody solution (Rabbit antimouse IgG-ALP conjugate) 1:2000 dilution, gently shaking for 1 hour.

A primary antibody for β-actin 1:30,000 dilution was also used as control for sample loading.

Antibody binding to nitrocellulose was detected using substrate solution BCIP/NBT (Bangalore Genei Pvt. Ltd. India) and bands were visible within 20 mins.

Results

OTC induced CAs, SCEs and frequency of MI in mouse BMCs and in HPBLs were studied as positive controls to either BSO+OTC or GSH+OTC treated samples and the data are presented in tabular form. Negative controls are data also shown.

In vivo

Cell cycle kinetics

The percentage of MI was significantly higher in OTC-treated samples at all fixation hours indicating a delay in cell proliferation. (Table 2.1) The AGT significantly increased after treatment with 15 mg kg^{-1} of OTC compared to control. Both the concentration of OTC showed almost similar extent of delay induction in cell proliferation. Presence of BSO reduces the delay induced by OTC (15 mg kg^{-1}) significantly, however with 30 mg kg^{-1}, presence of BSO induced more delay. Prior treatment of the animals with GSH reduced the delay considerably. It is worth noting, that BSO and GSH alone induced delay in cell proliferation with respect to negative control. (Table 2.2; Fig. 2.4)

CAs

The frequency of aberrant metaphases failed to increase by OTC treatment. Both 15 and 30 mg kg^{1} induced 3-4% aberrant metaphases. The aberrations were mainly chromatid break type and OTC failed to induced any exchange aberrations. The frequency of aberrant metaphases increased marginally when OTC was treated to BSO-treated mice, mainly the frequency of chromatid breaks were elevated. Treatment of GSH prior to OTC failed to reduce the frequency of OTC-induced CAs. BSO alone induced CAs significantly, however, the CAs induced by GSH alone was as good as that of the negative control. (Table 2.3; Fig.2.5)

SCEs

Table 2.4 shows that OTC induced significant level of SCEs in mouse BMCs in a dose dependent manner. However, in most of the studies 15 mg kg^{1} of OTC was used since at 30 mg kg^{1} several mice failed to survive. OTC at 15 mg kg^{1} induced more SCEs at 24h fixation cells than 20h fixed cells Treatment of OTC with GSH or BSO, increase the frequency of SCEs significantly. (Fig 2 5) It is also worth noting that both GSH and BSO alone induced SCEs considerably. Therefore, it is necessary to consider that both GSH and BSO per se induced a significant increase of 3.04 and 1.91 SCE/M respectively, with respect to untreated control. This means that considering an additive increase, if GSH+OTC induced an increase of 3.04+1 36 = 4 40 SCE/M, which compared with the observed SCE increase of 4 69, indicating a 6% increment. The data of the percent increment of the frequency of SCE/M in combined treatment is shown in Table 2.5. This enhancement was more pronounced in BSO + OTC-treated ones.

Regarding the distribution of SCEs per cell, it shows that OTC treatment showed more cells having 4-11 SCEs with respect to untreated control. In presence of BSO or GSH along with OTC, the cells having 8 and more SCEs increased substantially. The dispersion analysis indicated that the distribution of SCEs in OTC – treated with and without BSO and GSH did not deviate from the Poisson expectation, although GSH alone showed deviation from the Poisson expectation.

In vitro

Cell cycle kinetics

Unlike that of bone marrow cells, in HPBLs, the delay in cell kinetics induced by OTC is quite clear Table 2.6 shows that the frequency of M1 was significantly higher after OTC treatment in a dose dependent manner in most

of the experiments. Treatment of OTC to BSO-treated blood samples increased the frequency of MI cells and the increment was significant in all experiments, at an OTC concentration of 3μg ml^{-1}. However, both GSH and BSO alone showed significant increase in frequency of M1 indicating induction of delay in cell proliferation (Fig. 2.6).

CAs

Table 2.7 shows that the aberrant metaphases were significantly higher after OTC treatment in a dose dependent manner in most of the experiments. The aberrations observed were mostly chromatid breaks. Treatment of BSO increased the frequency of CAs, which was induced by OTC. When GSH was used prior to OTC treatment the frequency of OTC induced aberrant metaphases did not increase. It may be noted that BSO (5mM) and GSH (15mM) did not induce any kind of CAs since no change was observed from the frequency of spontaneous aberrations (Fig. 2.7)

SCEs

In HPBLs, OTC induces SCEs in a dose dependent manner. Presence of BSO before OTC treatment at 1μg ml^{-1} could not increase the frequency of SCEs and while at a concentration of 3μg ml^{-1}, not many cells were available to score SCEs since the frequency of MI cells are very high (Fig. 2.7). Presence of GSH 30 mins before OTC treatment showed marginal increment or decrement in the frequency of SCEs with respect to OTC-treatment alone. However, both GSH and BSO alone induced more SCEs with respect to control. The distribution of SCEs induced by both the doses of OTC with or without BSO or GSH was distributed as Poisson. The frequency of SCEs in the untreated controls did not differ significantly from each other and the dispersion of SCE was consistent with Poisson model (Table 2.8).

p53 protein expression

The purpose of this study was to examine the role of p53 protein in the cell delay process induced by OTC. Western blots were used to analyze the induction of the p53 protein after treatment with OTC. A marginal increase in the level of p53 protein was observed in mouse BMCs 6h post-treatment of OTC (Fig2.8).

The amount of p53 induced each time was determined after normalization to β-actin and was compared to the untreated controls.

Discussion

As outlined in the introduction, this study was carried out to evaluate the cytotoxic effect of OTC with respect to endogenous GSH level in mammalian cells. The present data indicate that OTC induced significant delay in cell kinetics and SCEs in both BMCs and HPBLs, however, with regard to CAs human lymphocytes showed higher induction than mouse BMCs. In this study OTC induced mostly chromatid breaks and failed to induce any exchanges. This implies that DNA lesions induced by OTC may not be appearing at the same time or in close proximity, so that they failed to associate to form exchanges (Kihlman 1977). Being structurally similar to cisplatin it is expected that the mode of action of the tin complexes could be similar to that of cisplatin (Crowe *et al* 1984). Thus it could be that OTC like cisplatin cross linked to the N7 position of two consecutive guanine molecules in DNA and disrupting the DNA helix. It has also been demonstrated that dimethyltin dichloride will form adducts with adenine, 9-methyl adenine and adenosine (Cardin and Roy 1985). Hence, it may be inferred that the OTC could bind on DNA more easily owing to its structural advantages particularly the increased bond length and thus possibly induces delay in cell proliferation and damages DNA.

A novel aspect of the present study is the analysis of the influence of BSO on OTC-induced CAs and SCEs. The data indicate that the presence of BSO increased cellular sensitivity towards OTC in both cell systems. Thus, BSO-mediated GSH depletion could lead more binding of OTC on DNA and increased its cytotoxicity. It was observed that some experimental mice failed to survive after OTC treatment and such death was increased when OTC was treated to BSO-treated mice. Although the exact reason for the death of mice by OTC is not known, it could be due to its neurotoxic potentiality, since organotin exhibits neurotoxic properties (Attahiru *et al* 1991). It seems that in the present study such toxic effect of OTC could probably be reduced when GSH is present. Treatment of BSO produces a rapid decrease in the GSH

levels of the various tissues (Lee *et al* 1987). Intermediate rates of depletion has been demonstrated following a single dose of 556 mg kg^{-1} BSO in the bone marrow with a nadir at 8-12h (Shrieve *et al* 1985). In an earlier study it has been observed that GSH concentration was depleted to 54% of the control value after 10h BSO treatment in mouse BMCs (Chattopahyay 1999). Therefore, the incubation period of BSO-treatment was kept for 10h in the present study and then OTC was applied. In the case of in vitro study the freshly drawn blood was incubated with BSO for 3h since in cultured cells >75% depletion was achieved within 4-5h by 500μm to 10mM BSO (Shrieve et al 1985, Edgren and Revesz 1987). In this study the concentration of BSO used was 5 mM since significant effect of BSO was observed at this concentration with respect to CA induction by radiation (Chattopahyay 1999) and bleomycin (Chattopahyay 1997). Depletion of cellular GSH by BSO or elevation of cellular GSH by exogenous GSH increased the induction of SCEs by OTC in BMCs, however, in HPBLs such influence was not observed. On the other hand, the frequency of CAs was increased significantly in both the systems when endogenous GSH level was depleted. It appears from the present result that BSO-mediated GSH depletion increases the number of DNA strand breaks induced by OTC, thereby enhancing the frequency of chromatid breaks only but not SCEs, particularly in case of HPBLs This observation indicates that DNA lesions for CAs and SCEs are qualitatively different as it was proposed earlier (Carrano *et al* 1979, Nishi *et al* 1984). It is also worth noting that BSO-mediated enhancement in DNA strand breaks failed to induce any exchange aberrations. We found that exogenous addition of GSH could not reduce the frequency of CAs in HPBLs and in case of BMCs the effect of GSH was not clear since the frequency of aberrations was very low. Such non-protective effect of GSH on OTC-induced CAs is not clear particularly when BSO-treatment enhanced the frequency of OTC-induced CAs It has been reported that addition of GSH to the culture media did not protect the cells from cell-death induced by triethyl- and trimethyltin in C6 glioma cells

(Cookson *et al* 1998). However, they observed protection when the culture was pretreated with 2-oxo-4-thiazolidine carboxylic acid, which increased intracellular GSH level. It seems that in the present study endogenous GSH level did not increase before addition of OTC since GSH is generally not considered to be transported easily across the cell membrane (Clark *et al* 1986) and very little enhancement in endogenous GSH level was shown after 2h of exogenous GSH addition in CHV79 cells (Wardman *et al* 1992). They had shown rapid increase in endogenous cysteine concentration (~10 fold) after exogenous GSH addition. Therefore, present failure in protection by exogenous GSH indicates the non-involvement of free radicals in OTC induced DNA-lesions since cysteine is more reactive than GSH towards radicals located on DNA (Zheng 1988).

It is also interesting to note that both GSH and BSO alone induced SCEs significantly in BMCs with respect to control as it was shown earlier (Chatterjee *et al* 1995). Due to the fact that this study considers the interaction between agents capable of inducing SCE, it is possible that each agent was capable of influencing the SCE-induction caused by other. However, the fact that the net SCE induction caused by OTC was enhanced in BMCs treated either with GSH and / or BSO, suggests that either GSH or BSO increases SCE induction by OTC. It seems that the mechanisms responsible for the present increased frequency of SCEs in two different combined treatments are not similar because of differences in cellular GSH status. Similar observation was made with mitomycinC in BMCs (Dev-Giri and Chatterjee 1998) where the role of GSH in the pattern of alkylation of DNA by MMC was known (Sharma and Tomasz 1994). In the present context, the role of GSH in OTC-DNA interaction is not known, however, it could be that when GSH was depleted by BSO, cross-linking of OTC with DNA could not be inhibited by GSH thus increased OTC efficacy was observed. Although one cannot rule out the possibility of SCE induction by peroxides and free radicals that are formed by normal pathway in BSO-treated cells as discussed earlier (Chatterjee *et al* 1995).

The frequency distribution of SCE / cell after OTC combined with either GSH or BSO showed Poisson distribution in both the systems which is an indication that the SCE-induction in those samples was due to damaging DNA since agents that induce SCEs by damaging DNA-lesions fit well with the Poisson distribution (Rainaldi and Mariani 1982). It was suggested that there are at least two forms of SCE induction. One is by damaging DNA (MacRae *et al* 1979) and another by inhibiting DNA synthesis (Ishii and Bender 1982). It seems that both forms of SCE induction could take place by OTC and the influence of endogenous GSH level on these lesions are different in these two systems Earlier evidence indicates that SCE is caused by DNA-lesions not repaired before the S-period, the cell cycle step in which the SCE seems to be produced (Perry and Wolff 1974). The reparability of the SCE-inducing lesions during G1 was not widely explored in vitro (He and Lambert 1985) and less in vivo (Morales-Ramirez et al 1995); the latter is due to the difficulties in obtaining synchronized cells. It is most likely that many SCE-inducing DNA lesions could be repaired during G1 in HPBLs which is perhaps not true for dividing BMCs. Repair of 50% of the SCE-inducing lesions during G1 caused by MNU was observed by liquid holding experiment in HPBLs (Stephenou *et al* 1996). From the present data it seems that the SCE-inducing lesions caused by OTC are qualitatively different in BMCs and HPBLs and endogenous GSH level perhaps exerts its influence on some of these lesions. Although it is not clear, exactly which types of lesions induced SCEs in this two different systems, but their seems to be different types as it was proposed earlier about the existence of various types of SCE-inducing lesions (Saher *et al* 1981; Kano and Fujiwara 1982) on which some are repairable and others are nonrepairable (Stephenou *et al* 1996; Gonzales-Beltran 1999).

In this study OTC induced significant delay in cell cycle in both the systems. In vivo, although the basic cell cycle progression varied considerably among individuals in each group, the extent of delay by OTC was very clear. The AGT was significantly increased after treatment with 15

mg kg^{-1} of OTC compared to control. Treatment with BSO or GSH along with OTC reduced the delay in cell cycle significantly and this delay reduction was more pronounced with GSH. However, with 30 mg kg^{-1}, the results obtained were quite unusual. As mentioned earlier in the introduction (page 8), The concentration of OTC is an important factor for its toxicity. At 30 mg kg^{-1} 40% of the animals failed to survive and this increased further when BSO was treated prior to OTC – treatment. This could be the reason of the quirk observation made at 30 mg kg^{-1} OTC - treatment. Other Organotin compounds are known to inhibit the DNA synthesis of spleen cells in mice (Al-Imara *et al* 1993) and decreased the proliferation of human B lymphocytes (De Santiago *et al* 1999). It could be that in this study OTC form an adduct with DNA and interfere with DNA replication and induce cell cycle delay. However, present data indicate that OTC-DNA adduct may not be the sole factor for inducing delay in cell cycle, because in BMCs, BSO increased OTC-induced CAs but reduced delay in cell cycle and in HPBLs, GSH reduced OTC-induced delay in cell kinetics but not to CAs. It has been reported that di- and trisubstituted organotins affect cell energetic, membrane-associated functions and macromolecular synthesis (Caruso *et al* 1993). Therefore, it is difficult to say the exact mechanism by which OTC induced delay in cell cycle. It is reasonable to believe that not one single event is responsible for mutagen-induced delay in cell cycle rather it may be dependent on multivariate factors.

GSH alone induces a delay in cell kinetics in both the systems as it was shown earlier (Chatterjee *et al* 1986; Chatterjee *et al* 1995; Speit 1980). In general aminothiol radioprotectors are thought to bind with DNA and slow down strand separation for replication (Brown 1967). However, in this study BSO alone induces delay in BMCs but not in HPBLs. It could be that the generation of free radicals might be negligible in non-cycling lymphocytes (before PHA addition) and thus depletion of cellular GSH by BSO could not induce delay in cell cycle as well as CAs. This is not true for cycling BMCs where depletion of GSH failed to protect DNA from peroxides and free

radicals, which are formed by normal metabolic pathways, and induce delay in cell kinetics and CAs.

In the present study we had measured the level of p53 only after 6h of OTC-treatment. It was observed that there is slight increase in p53 protein with respect to control level. In fact, one should measure every 2h interval for 20h after OTC-treatment to get a clear picture of the p53 – level. From the present study it seems that OTC-induces delay in cell cycle in a p53-dependent manner. In normally growing cells p53 is rapidly turned over and protein levels are very low. In response to stress signals such as DNA damage, hypoxia, mitotic spindle damage or oncogene activation, p53 is stabilized and protein levels increase rapidly (Ashcroft and Vousden 1999). One of the principal regulators of p53 activity is MDM2, which binds directly to the N-terminus of p53. The cellular concentration of p53 protein increases in response DNA-damage. Protein stabilization may also contribute to the rise in p53 protein concentration in response to DNA-damage (Milner 1996). The importance of p53 in normal cell growth is supported by the fact that its function is lost in approximately half of all human cancers. It involved in several aspects of cell cycle, apoptosis, control of genome integrity and DNA repair (Levine 1997). p53 influences proliferation by acting predominately in the G1 phase of the cell cycle progression. Oncogenic, DNA-damage by irradiation and chemotherapeutic drugs activate p53 (Levine1997). p53 causes a G1 arrest by inducing expression of p21, numerous other studies demonstrate that p53 also functions at G2/M checkpoint. Quite frequently in many mouse, rat and human cell lines over-expression of p53 inhibits entry into mitosis (Agarwal 1998). This checkpoint seems to be activated when DNA synthesis is blocked and prevents segregation of damaged or incompletely synthesized DNA. p53 functions are mediated by direct interactions with other proteins, or with the DNA (Kastan 1991; Kuerbitz 1992). Exposure to radiation leads to an increase in the levels of proteins that derives from an alteration in its half-life as a result of post-translational modifications (Levine 1997; Ko and Prives 1996). Similar observation was

also obtained with exposure to bleomycin and other chemotherapeutic drugs (Lu and Lane 1993). The tumor-suppressor protein p53 plays a role in determining the resistance of cancer cells to chemotherapeutic agents (Linn et al 1996).

Several reports show that organotin compounds seem to have a vast potential for use as antitumour agent (Crowe *et al* 1984; Saxena and Huber 1989). Unfortunately, even for the most widely used anticancer drugs, there are gaps in the literature that make it difficult to assess the relative mutagenic potential of different types of compound (Ferguson 1995). The present novel tin compound shows ability in blocking cell proliferation and damaging DNA in mammalian cells and this effect exacerbates further when cellular GSH level is depleted by BSO. Since the cytotoxic effect of OTC is more in GSH depleted cells, the concentration of OTC may be reduced to get an antitumour effect in GSH-depleted cells and thus minimizes its toxic side effect.

It has been shown that the effect of GSH on the cytotoxicity of cisplatin and iproplatin in a human melanoma cell line was not a consequence of differences in GSH-Pt conjugation, but rather that it could be attributable to something else, such as DNA-repair, apoptosis and free radical scavenging or other unknown mechanisms (Pendyala *et al* 1997). Therefore, successful evaluation of new anticancer drugs increasingly depends on a detailed understanding of mechanism of action, its cytotoxicity and pharmacology.

Table.2.1. Effect of OTC alone or in combination with BSO (200mg kg^{-1}) and GSH (400mg kg^{-1}) on the cell cycle kinetics in mouse bone marrow cells *in vivo*.

Exp. condition	Fixation time	TM	M1%	M2%	AGT	Mean AGT ± SEM
Untreated	17	302	49	47	10.62	
		160	34	60	08.13	
		204	57	39	14.82	
		394	43	55	12.96	11.63 ± 1.44
Untreated	20	178	08	62	08.56	
		080	28	52	09.92	
		156	24	50	09.62	
		130	29	53	09.62	09.43 ± 0.65
Untreated	24	196	09	78	11.92	
		163	10	70	12.00	
		142	15	65	12.37	12.09 ± 0.14
BSO	20	352	45	55	13.83	
		287	51	49	14.82	14.32 ± 0.50
GSH	20	149	23	59	09.82	
		231	43	55	11.66	
		218	61	39	13.85	
		160	38	57	11.19	11.63 ± 0.84
OTC 15mg kg^{-1}	17	403	94	06	16.20	
		299	90	10	15.60	
		246	89	11	15.90	
		102	90	10	16.10	15.95 ± 0.13
BSO+OTC 15mg kg^{-1}	17	211	85	15	17 90	
		458	66	22	15.25	16.55 ± 1.35
OTC 15mg kg^{-1}	20	253	78	19	16.80	
		306	65	33	15.10	
		276	88	12	18.32	
		166	71	29	15.85	
		134	63	37	15.00	16.20 ± 0.62
BSO+OTC 15mg kg^{-1}	20	326	38	61	12.68	
		406	45	67	13.20	
		135	78	22	16.80	14.20 ± 1.31
GSH+OTC 15mg kg^{-1}	20	150	22	78	11.55	
		207	32	68	12.23	
		328	63	37	14 84	
		263	22	75	11.80	
		234	23	75	11.52	12.24 ± 0.66
OTC 15mg kg^{-1}	24	170	30	66	13.86	
		101	32	68	14.60	
		227	17	77	12.94	
		156	25	66	13.61	13.76 ± 0.49
OTC 30mg kg^{-1}	17	310	82	18	14.80	
		241	81	19	14.35	14.55 ± 0.25
OTC 30mg kg^{-1}	20	354	63	37	14.30	
		274	40	52	12.23	
		275	44	54	12.95	
		213	30	68	12.25	12.85 ± 0.52
BSO+OTC 30mg kg^{-1}	20	125	100	00	20.50	
		180	100	00	20.50	
		102	097	03	20.36	20.26 ± 0.13

TM : Total Metaphases; M1 : 1st cycle Metaphases; AGT : Average Generation Time

Table2.2. Effect of OTC (mg kg^{-1}) alone or along with BSO (200mg kg^{-1}) and GSH (400mg kg^{-1}) on cell kinetics in mouse bone marrow cells in vivo.

Exp. condition	Fixation time(h)	TM	M1%	Range of M1%	AGT(h)±SEM
Untreated	17	1060	46	34-57	11.63 ± 0.56
OTC(15)		1050	91[$]	89-94	15.95* ± 0.13
BSO+OTC		0669	76	66-85	16.55 ± 1.35
OTC(30)		0551	82	81-82	14.55 ± 0.25
Untreated	20	0549	22	08-29	09.43 ± 0.48
BSO		1116	27	06-51	09.98 ±1.00
GSH		0758	41	23-61	11.60 ± 0.80
OTC(15)		1135	73[$]	63-88	16.20* ± 0.61
BSO+OTC(15)		0867	44	38-78	14.20 ± 1.31
GSH+OTC		1182	32	22-63	12.24 ± 0.66
OTC(30)		1116	44	30-63	12.85 ± 0.52
BSO+OTC(30)		0407	99[$]	97-100	20.26 ± 0.13
Untreated	24	0501	11	09-15	12.09 ± 0.14
BSO		1325	11	06-14	12.47 ± 0.23
OTC		0654	26[$]	17-32	13.76 ± 0.49

*$p<0.01$; Student's t-test, compared to respective control.
[$] $p< 0.01$; 2x2 contingency χ^2 test, compared to respective control.

Table 2.3. Effect of OTC alone or along with BSO (200 mg kg^{-1}) and GSH (400mg kg^{-1}) on chromosomal aberrations in mouse BMCs *in vivo.*

Expt. condition	Fixation time	TM	Ab.M%	Mean ±SEM	Aberration % chd.bk	Mean ±SEM	Iso.chd.bk	Mean ±SEM
Untreated	20	108	01		01		00	
		101	01		01		00	
		102	02		01		01	
		133	02	02 ± 0.25	02	01 ± 0.25	01	01± 00
BSO	20	134	09		09		01	
		136	09		10		02	
		097	14		10		02	
		103	06	10[a] ± 1.22	07	09 ± 0.70	00	02 ± 0.48
GSH	20	112	03		03		01	
		109	02	03 ± 0.35	01	02 ± 0.71	01	01 ± 00
OTC 15mgkg^{-1}	17	207	03		02		01	
		145	04		04		00	
		163	04		03		01	
		059	00	03 ± 0.94	00	02 ± 0.85	00	01 ± 0.28
BSO+OTC 15mgkg^{-1}	17	114	04		03		02	
		184	07	05 ± 0.73	06	05 ± 1.06	01	02 ± 0.35
OTC 30mgkg^{-1}	17	178	03		02		01	
		150	04	04 ± 0.42	04	03 ± 0.70	00	01 ± 0.50
OTC 15mgkg^{-1}	20	128	02		02		00	
		103	03		02		01	
		201	04		03		01	
		069	03		03		00	
		060	02	03 ± 0 37	02	02 ± 0.25	00	0.4 ± 0.24
BSO+OTC 15mgkg^{-1}	20	078	06		05		01	
		126	09		07		02	
		082	04	06 ± 0.53	03	05 ± 1.15	01	02 ± 0.33
GSH+OTC 15mgkg^{-1}	20	056	04		03		01	
		078	04		02		02	
		174	04		03		01	
		089	05		05		00	
		076	04	04 ± 0.18	04	04 ± 0.28	00	01 ± 0.21
OTC 30mgkg^{-1}	20	187	03		03		00	
		089	02		01		01	
		090	04		04		00	
		056	04	03 ± 0.53	04	03 ± 0.70	00	0.25 ± 0.25
BSO+OTC 30mgkg^{-1}	20	105	09		07		02	
		150	13		11		02	
		075	09	10[a] ± 0.51	07	08 ± 1.33	02	02 ± 00

TM: Total metaphases for chromosomal aberration.
[a] $p<0.001$;2x2 contingency χ^2- test compared to control.

Table 2.4. Details of individual SCE values induced by OTC alone or in combination with BSO and GSH, distribution of SCEs and dispersion coefficient in mouse bone marrow cells *In vivo*.

Expt. condition	Fixation time	TM	SCE/M	Mean ± SEM	Dispersion % 0-3	4-7	8-11	12>	$H^{\$}$
Untreated	20	37	2.37		69	29	02	00	1.21
		35	2.77		58	40	02	00	1.34
		65	2.35		65	30	05	00	1.45
		41	2.90	2.60 ± 0.12	60	35	05	00	1.32
Untreated	24	68	3 60		68	31	02	00	0.83
		44	3.20		56	38	06	00	1.38
		50	3.40	3.40 ± 0.12	52	41	07	00	0.94
BSO	20	59	4.47		35	42	21	02	0.98
		51	4.55	4.51 ± 0.03	40	45	15	00	1.04
GSH	20	42	5.14		30	52	18	00	1.58@
		55	6.36		45	45	10	00	1.75@
		60	6.32		27	53	15	05	1.69@
		46	4.72	5.64 ± 0.36	38	49	11	01	1.48
OTC 15 $mgkg^{-1}$	20	32	3.57		50	44	06	00	0.89
		45	3.45		58	36	06	00	1.74@
		25	3.80		40	60	00	00	0.57
		26	4.25		23	54	23	00	1.00
		27	4.75	3.96 ± 0.24	20	63	17	00	0.74
BSO+OTC 15 $mgkg^{-1}$	20	114	6.48		18	48	27	07	1.67@
		164	7.20		12	42	37	09	1.42
		076	7.10	6.93 ± 0.22	13	45	36	06	0.87
GSH+OTC 15 $mgkg^{-1}$	20	75	6.28		12	77	11	00	0.58
		87	7.61		07	47	35	11	1.35
		58	7.13		06	47	45	02	0.86
		79	7.80	7.29 ± 0.28	07	38	43	12	1.42
OTC 15 $mgkg^{-1}$	24	79	5.87		16	58	23	03	1.04
		31	6.60		07	52	39	04	0.52
		77	7.42		05	52	39	04	0.52
		75	6.63	6.63 ± 0.45	05	52	42	03	0.76
OTC 30 $mgkg^{-1}$	20	85	4.50		26	68	06	00	0.69
		68	5 30		19	63	13	05	0.75
		71	4.48		35	52	11	02	1.39
		94	5.78	5.02 ± 0.32	20	59	19	02	1.06

TM : Total Metaphases; $H^{\$}$: dispersion coeffecient = variance/mean.
@ Significantly different at α = 0.05 from Poisson distribution.

Table 2.5. Effect of OTC (15mg kg^{-1}) alone or along with BSO (200mg kg^{-1}) and GSH (400mg kg^{-1}) on SCEs in mouse BMCs in vivo.

Exp. condition	TM	Mean SCEs ±SEM	Distribution(%) 0-3	4-7	8-11	12>	H[$]	SCE increased observed*	expected**	increment%
Untreated	178	2.6 ± 0.1	63	33	04	00	0.95			
BSO	110	4.5 ± 0.0[a]	37	44	18	01	1.01	1.91		
GSH	203	5.6 ± 0.4[a]	35	50	13	02	1.62[@]	3.04		
OTC	155	4.0 ± 0.2[b]	38	51	11	00	0.98	1.36		
BSO+OTC	354	7.0 ± 0.2[b]	14	45	33	08	1.32	4.33	3.27	32
GSH+OTC	378	7.3 ± 0.3[a]	08	50	35	07	1.07	4.69	4.40	06

TM : Total Metaphases scored for SCEs.
*Observed data-untreated value; **Additive effect of each agent – untreated value.
[$]H: dispersion coefficient = variance/mean.
[a]$p<0.01$; [b]$p<0.05$; Student's t-test, compared to respective control.
@ Significantly different at $\alpha = 0.05$ from Poisson distribution.

Table 2.6. Effect of OTC on cell kinetics in human peripheral blood lymphocytes *in vitro* alone or along with BSO (5mM) and GSH (15mM).

Experimental condition	TM	M1%	M2%	AGT (hours)	Donor #
Untreated	234	41	41	40.4	1
1μg ml^{-1}	173	55[a]	44	49 3	
3	123	64[b]	36	53.1	
Untreated	180	45	47	40.0	2
3μg ml^{-1}	213	64[a]	35	52.5	
BSO+3	136	88[b]	12	53.1	
Untreated	242	46	45	45.0	3
3μg ml^{-1}	178	78	22	60.0	
BSO+3	097	98	02	71.0	
Untreated	168	43	46	45.0	4
1μg ml^{-1}	170	61	38	51.4	
BSO+1	162	67	33	55.3	
Untreated	132	54	35	48.0	5
1μg ml^{-1}	179	61	37	51.4	
3	157	68[a]	32	55 4	
BSO+1	157	66	32	55.3	
BSO+3	082	80[a]	20	65 4	
Untreated	168	48	42	45.0	6
GSH	183	52	32	41.6	
BSO	155	49	39	44.2	
1μg ml^{-1}	118	60[a]	37	51.4	
3	144	80[b]	20	65.4	
Untreated	127	43	53	45.0	7
GSH	214	57[a]	36	44.5	
BSO	120	46	42	45.0	
1μg ml^{-1}	165	59[a]	36	51.4	
3	133	62[a]	38	55.3	
BSO+1	124	63	37	55.3	
BSO+3	095	75[a]	25	60.0	
Untreated	255	52	43	48.0	8
GSH	122	65[a]	30	51 4	
BSO	130	69[a]	20	50.7	
1μg ml^{-1}	189	61	37	50 1	
3	123	64[a]	36	53.1	
BSO+1	132	85[b]	15	62 6	
BSO+3	125	96[b]	04	69.2	
GSH+1	188	63	37	55 4	
GSH+3	215	65	35	53.4	

TM: Total Metaphases for cell kinetics; M1: 1st cycle Metaphases
AGT: Average generation time.
[a] $p< 0.05$; [b] $p< 0\ 01$; χ^2 – Test compared to their respective control.

Table 2.6. Effect of OTC on cell kinetics in human peripheral blood lymphocytes *in vitro* alone or along with BSO (5mM) and GSH (15mM).

Experimental condition	TM	M1%	M2%	AGT (hours)	Donor #
Untreated	214	57	35	48.3	9
1μg ml^{-1}	179	62	38	51.4	
3	158	68[a]	32	55.4	
BSO+1	112	73[a]	27	57.6	
BSO+3	100	100[b]	00	72.0	
GSH+1	121	62	38	52.1	
GSH+3	117	60	40	51.6	
Untreated	205	59	35	48.6	10
1μg ml^{-1}	179	61	37	51.1	
3	157	68	32	54.9	
GSH+1	155	61	39	51.8	
GSH+3	136	68	32	54.5	
Untreated	195	51	41	48.0	11
1μg ml^{-1}	178	62[a]	35	51.4	
BSO+1	112	75[a]	25	57.6	
GSH+1	122	66	34	53.7	
Untreated	236	52	38	45.5	12
1μg ml^{-1}	182	63[a]	33	50.8	
3	178	74[b]	26	57.1	
GSH+1	177	64	32	51.6	
GSH+3	184	65	31	50.7	

TM: Total Metaphases for cell kinetics; M1: 1st cycle Metaphases
AGT: Average generation time.
[a] $p < 0.05$; [b] $p < 0.01$; χ^2 – Test compared to their respective control.

Table 2.7. Effect of OTC alone or along with BSO (5mM) and GSH (15mM) on chromosomal aberration (CAs) in HPBLs *in vitro*.

Experimental condition	TM	% Ab M	Aberration % chd	bk	del	Donor #
Untreated	068	02	02	--		1
1μg ml^{-1}	084	06	06	--		
3	075	13[b]	12	01		
Untreated	073	03	03	--		2
3μg ml^{-1}	126	11[b]	11	--		
BSO + 3	111	11	11	--		
Untreated	085	02	02	--		3
3μg ml^{-1}	103	14[b]	12	02		
BSO + 3	061	16	14	02		
Untreated	063	02	02	--		4
1μg ml^{-1}	103	06	06	--		
BSO + 1	109	07	06	01		
Untreated	054	02	02	--		5
1μg ml^{-1}	101	08	08	--		
3	103	10[a]	10	--		
BSO + 1	082	09	09	--		
BSO + 3	062	15	15	--		
Untreated	136	01	01	--		6
GSH	083	01	01	--		
BSO	085	02	02	--		
1 μg ml^{-1}	132	11[b]	11	--		
3	100	16[b]	14	02		
Untreated	100	02	02	--		7
GSH	080	02	02	--		
BSO	073	02	02	--		
1 μg ml^{-1}	087	04	04	--		
3	074	16[b]	16	--		
BSO + 1	091	13[a]	13	--		
BSO + 3	095	21	21	--		
Untreated	114	02	02	--		8
GSH	059	03	03	--		
BSO	069	02	02	--		
1 μg ml^{-1}	098	11[b]	11	--		
3	101	14[b]	14	--		
BSO + 1	080	14	14	--		
BSO + 3	096	19	19	--		
GSH + 1	093	03	03	--		
GSH + 3	119	13	13	--		

TM, Total Metaphases, Ab M, Aberrant Metaphases

[a] $p< 0.05$, [b] $p<0.01$, 2x2 contingency χ^2 - test with respect to their respective control

Table 2.7. Effect of OTC alone or along with BSO (5mM) and GSH (15mM) on chromosomal aberration (CAs) in HPBLs *in vitro*.

Experimental condition	TM	% Ab M	Aberration % chd	bk	del	Donor #
Untreated	085	01	01	--		9
1μg ml^{1}	091	10	10	--		
3	100	14[b]	14	--		
BSO + 1	103	12	12	--		
BSO + 3	084	17	15	02		
GSH + 1	072	08	08	--		
GSH + 3	079	14	14	--		
Untreated	099	01	01	--		10
1 μg ml^{1}	102	07	07	--		
3	105	11[a]	10	01		
GSH + 1	080	06	06	--		
GSH + 3	083	12	11	01		
Untreated	112	02	02	--		11
1 μg ml^{1}	082	09[a]	09	--		
BSO + 1	062	13	13	--		
GSH + 1	067	05	05	--		
Untreated	117	03	03	--		12
1 μg ml^{1}	104	06	06	--		
3	072	08	07	01		
GSH + 1	097	07	06	01		
GSH + 3	104	12	10	02		

TM, Total Metaphases, Ab M, Aberrant Metaphases
[a] p< 0 05, [b] p<0 01, 2x2 contingency χ^2 - test with respect to their respective control

Table 2.8. Induction of SCEs by OTC in HPBLs *in vitro* along with BSO (5mM) and GSH (15mM).

Experimental condition	TM	SCE/M	Distribution % 0-3	4-7	8-11	12>	$H^{\$}$	Donor #
Untreated	47	6.5	13	55	30	02	0.48	1
1μg ml^{-1}	37	8.5[a]	00	43	38	19	0.79	
3	25	9.6[b]	00	14	59	27	0.63	
Untreated	40	5.8	13	73	15	13	0.45	2
3μg ml^{-1}	45	8.1[b]	07	33	51	08	0.67	
BSO+3	08	8.8	00	63	25	13	2.40	
Untreated	55	5.8	13	69	13	06	0.66	3
3μg ml^{-1}	25	7.4[a]	00	52	40	08	0.43	
Untreated	36	5 0	20	72	08	00	0.79	4
1μg ml^{-1}	31	7.5[a]	07	52	36	06	0.87	
BSO+1	27	7.6	00	52	44	04	0.55	
Untreated	29	5.7	21	59	17	03	1.68[@]	5
1μg ml^{-1}	30	7.6[a]	03	47	50	00	0.52	
3	23	8.6[b]	00	48	52	00	0.71	
BSO+1	25	7.8	04	32	60	04	0.82	
BSO+3	07	9.5	00	14	57	29	0.87	
Untreated	35	5.4	20	57	23	00	0.80	6
GSH	37	5.3	13	43	28	16	1.24	
BSO	88	5.6	19	51	21	09	1.37	
1μg ml^{-1}	23	6.5[a]	09	61	22	09	0.90	
3	27	8.8[b]	04	33	42	21	1.00	
Untreated	27	5.4	15	78	07	00	0.63	7
GSH	26	6.1[a]	07	69	17	04	1.94[@]	
BSO	30	6.2[a]	14	50	29	07	1.63[@]	
1μg ml^{-1}	35	7.4[a]	06	54	37	03	0.71	
3	25	8.0[a]	04	52	36	08	1.09	
BSO+1	22	7.1	04	52	37	07	0.62	
BSO+3	14	8.1	00	36	50	14	0.71	
Untreated	46	5 2	17	74	09	00	0.58	8
GSH	49	7.8[a]	12	41	09	00	0.53	
BSO	88	6.4[a]	17	49	29	05	1.25	
1μg ml^{-1}	39	8.5[b]	00	45	36	20	0.79	
3	25	9.8[b]	00	13	58	29	0.63	
BSO+1	12	8.5	00	75	13	13	1.19	
GSH+1	40	7.4	05	45	50	00	0.61	

TM ; Total Metaphases. $H^{\$}$; Dispersion co-efficient = variance/mean
[a] $p< 0\ 10$; [b] $p < 0\ 05$; Student's t-test compared to their respective control.
[@] Significantly different at $\alpha = 0\ 05$ from Poisson distribution

Table 2.8. Induction of SCEs by OTC in HPBLs *in vitro* along with BSO (5mM) and GSH (15mM).

Experimental condition	TM	SCE/M	Distribution % 0-3	4-7	8-11	12>	$H^{\$}$	Donor #
Untreated	39	5 9	13	62	21	00	0.66	9
1μg ml^{-1}	32	6 7	06	59	31	03	0.69	
3	33	8.3[a]	00	40	50	10	0.71	
BSO+1	20	8.0[a]	00	35	65	00	0.26	
GSH+1	30	7.2	07	50	40	03.3	0.59	
GSH+3	31	8.5	00	31	50	19	0.61	
Untreated	37	5 8	16	74	09	02	0.66	10
1μg ml^{-1}	30	7 6[b]	03	55	34	06	0.71	
3μg ml^{-1}	25	7.8[b]	00	33	56	10	1.02	
GSH+1	25	7.2	00	57	39	04	0.59	
GSH+3	21	8.5	00	37	47	16	0.81	
Untreated	32	5 6	19	72	09	00	0.57	11
1μg ml^{-1}	39	8.8[a]	00	43	38	19	0.79	
BSO+1	21	9 6	05	22	43	29	1 12	
GSH+1	23	7.5	00	52	43	04	0.65	
Untreated	42	5 5	21	60	19	00	0.71	12
1μg ml^{-1}	34	7.1[a]	00	44	44	11	0.82	

TM : Total Metaphases. $H^{\$}$; Dispersion co-efficient = variance/mean

[a] $p < 0.10$; [b] $p < 0.05$; Student's t-test compared to their respective control.

@ Significantly different at $\alpha = 0.05$ from Poisson distribution.

Fig. 2.1: Microphotographs showing Hoechst-Sunlight-Giemsa staining pattern of chromosomes in mouse bone marrow cells in the presence of BudR.

A. Normal one division cycle.

B. Second division cycle (→) showing sister chromatid exchange (SCE).

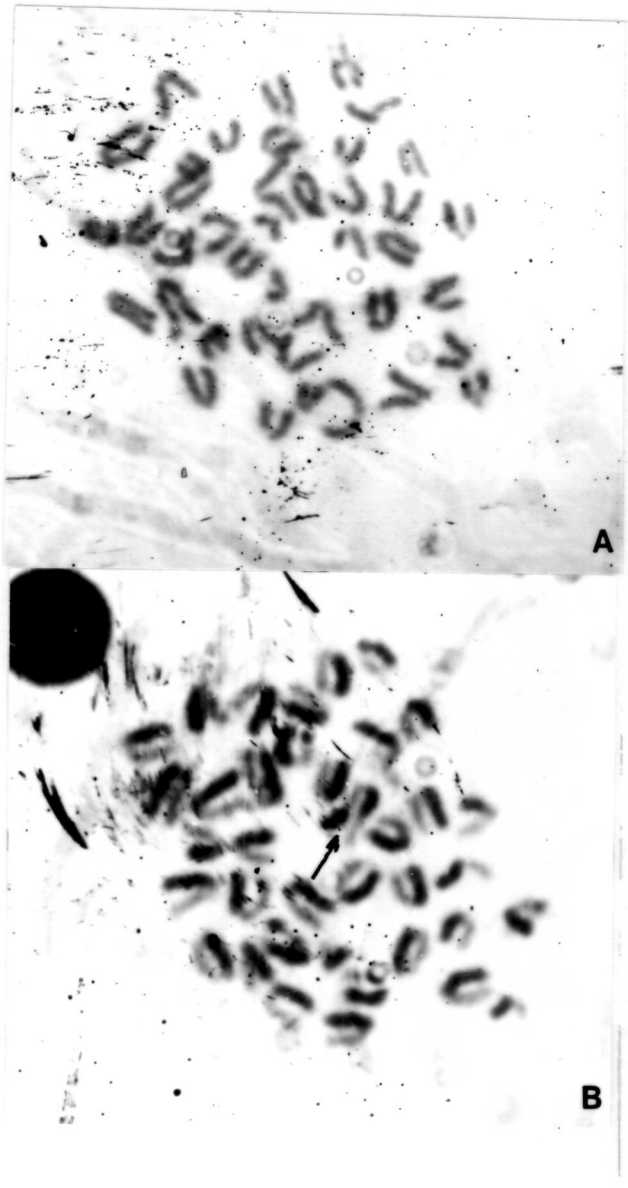

A
B

Fig. 2.2: Microphotographs showing Hoechst-Sunlight-Giemsa staining pattern in human peripheral blood lymphocytes in presence of BudR.

A. Normal one division cycle.

B. Second division cycle (→) showing sister chromatid exchange (SCE).

C. Third division cycle.

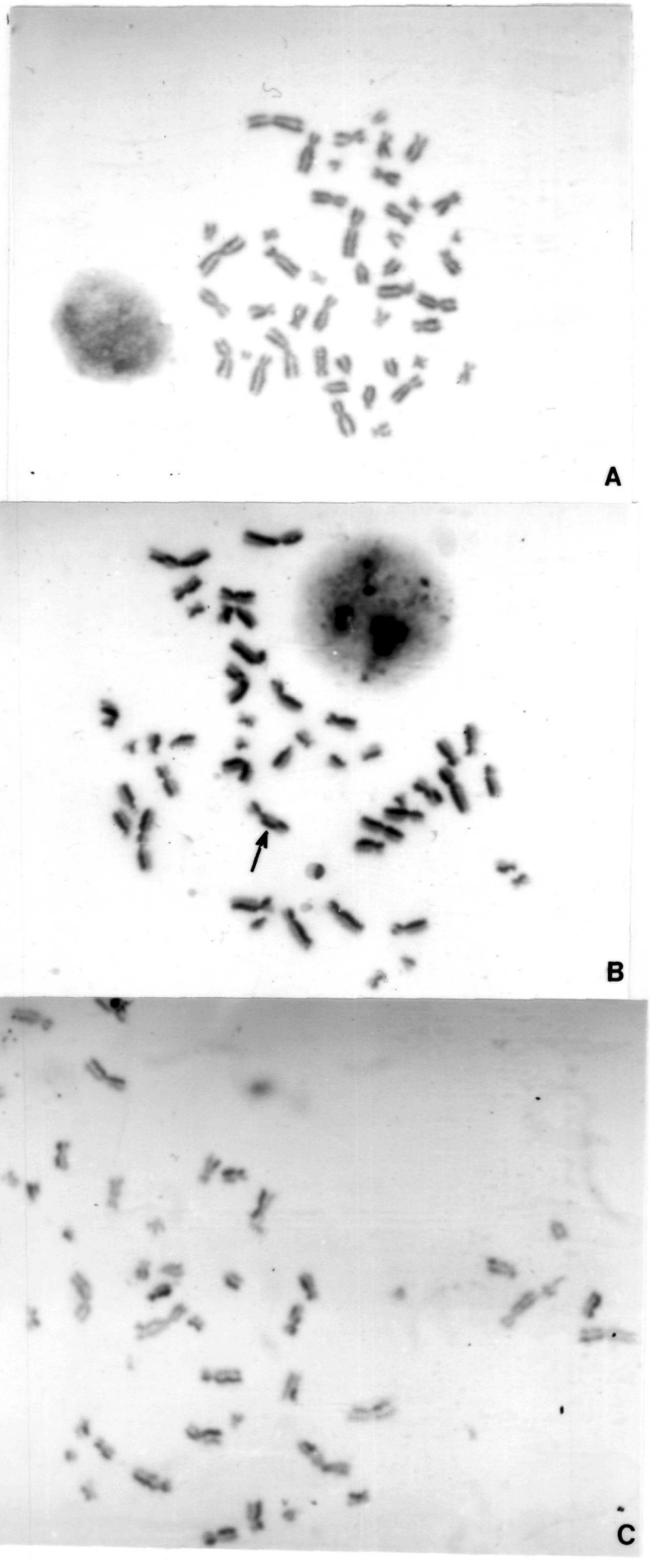

A
B
C

Fig.2.3: Microphotograph showing OTC induced aberration in human peripheral blood lymphocytes.

A. One division cycle showing chromatid aberration (→).

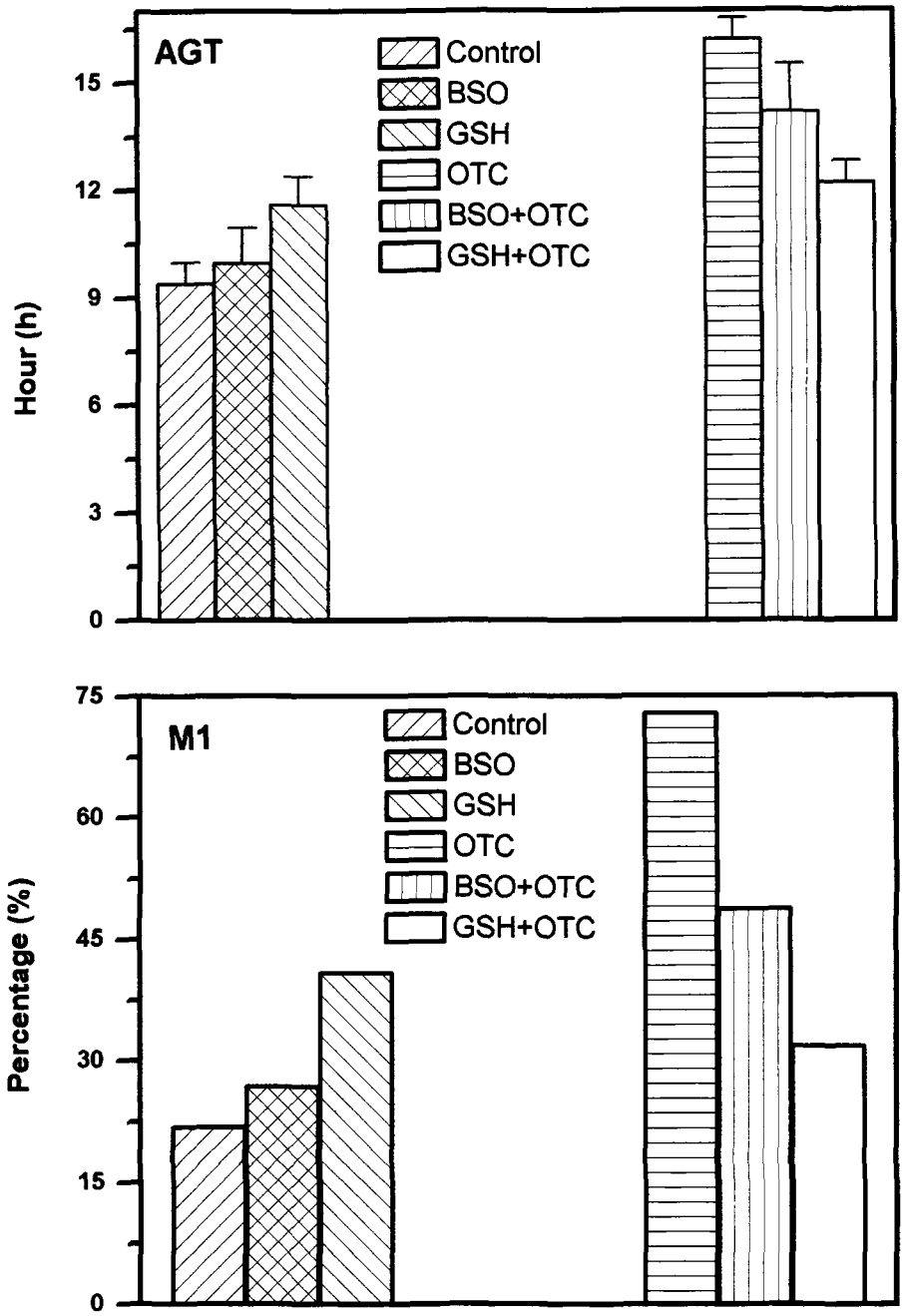


Fig. 2.4

Effect of OTC (15 mg kg^{-1}) with or without BSO and GSH on the frequency of AGT and M1 percentage in mouse BMCs. The plotted values are the means± SEM.

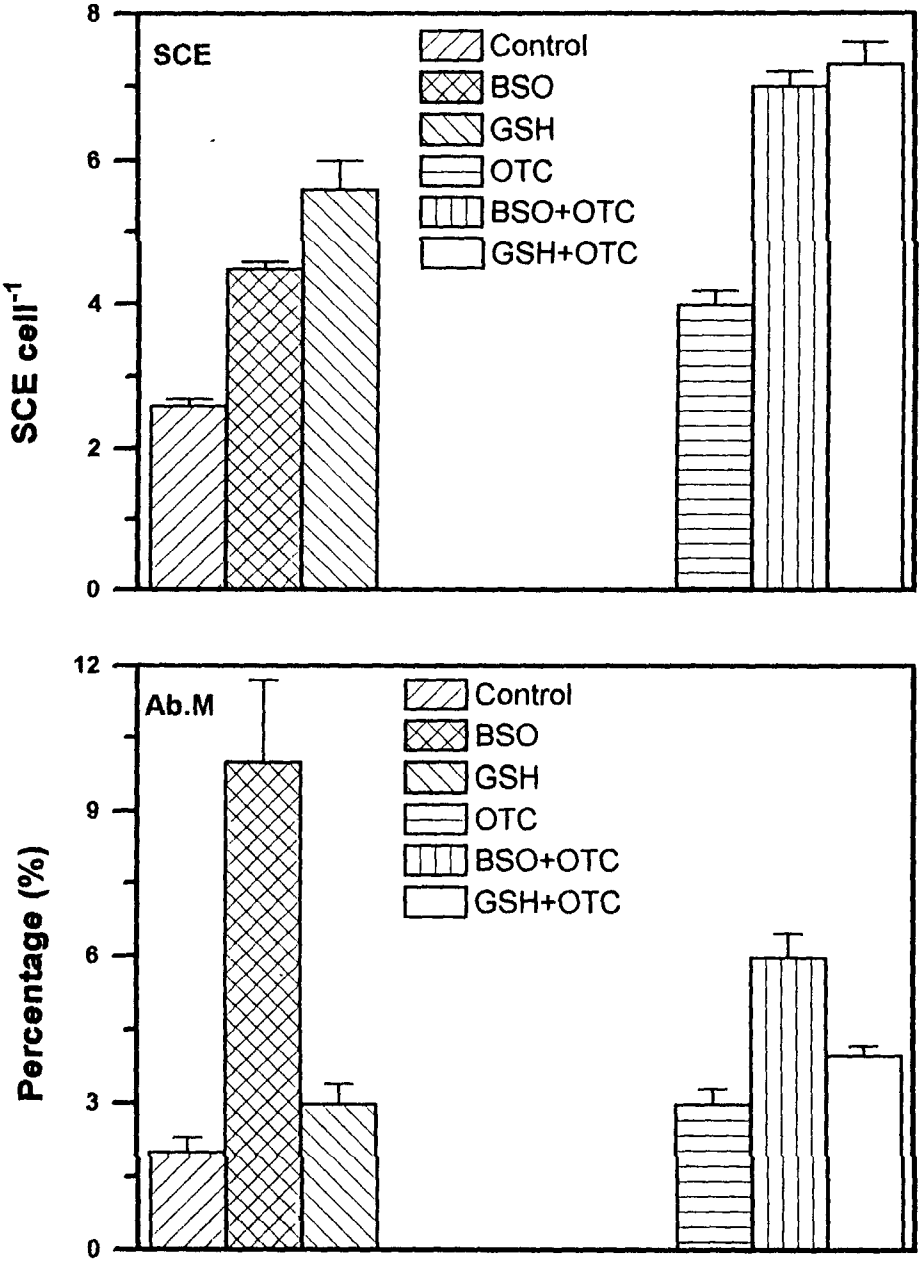


Fig. 2.5

Effect of OTC (15 mg kg^{-1}) with or without BSO and GSH on the frequency of aberrant metaphase and SCEs in mouse BMCs. The plotted values are the means ± SEM.

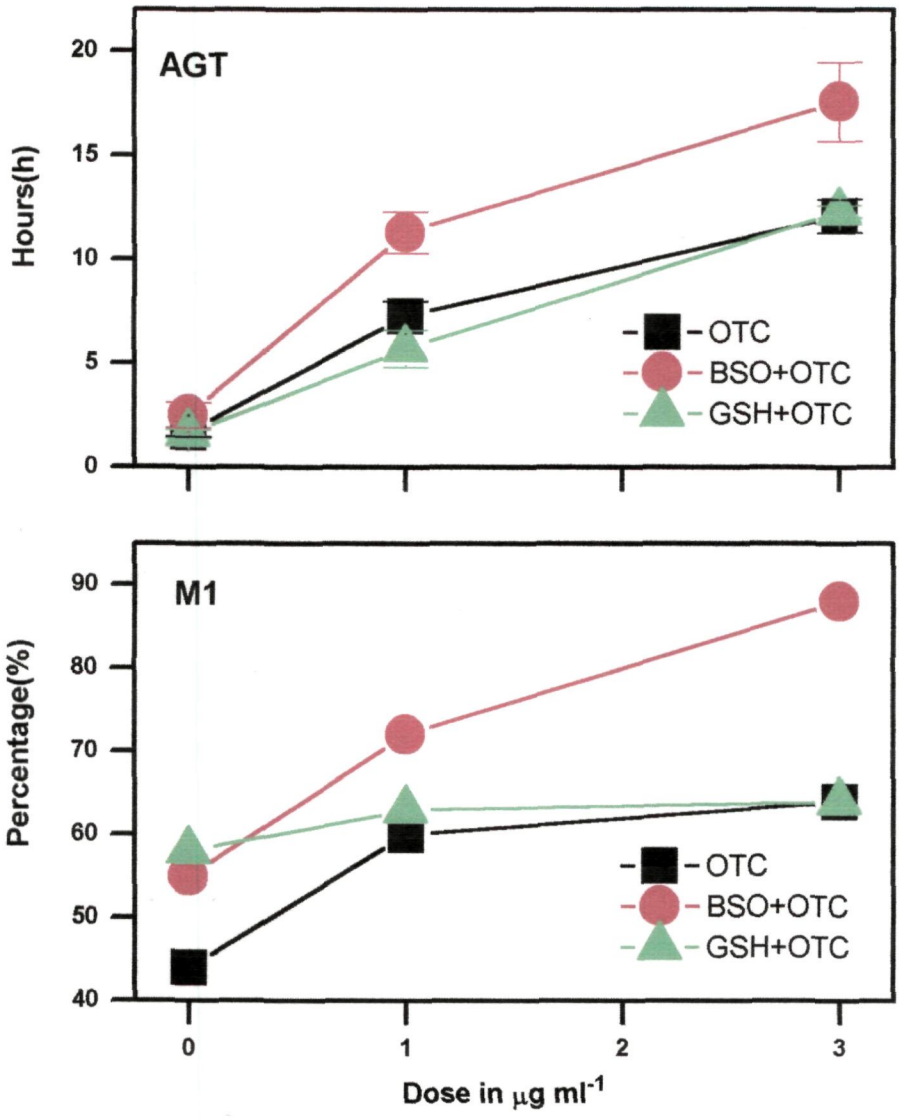


Fig. 2.6

Effect of OTC with or without BSO and GSH on the frequency of M1 and duration of AGT in HPBLs. The plotted values are the mean ± SEM.

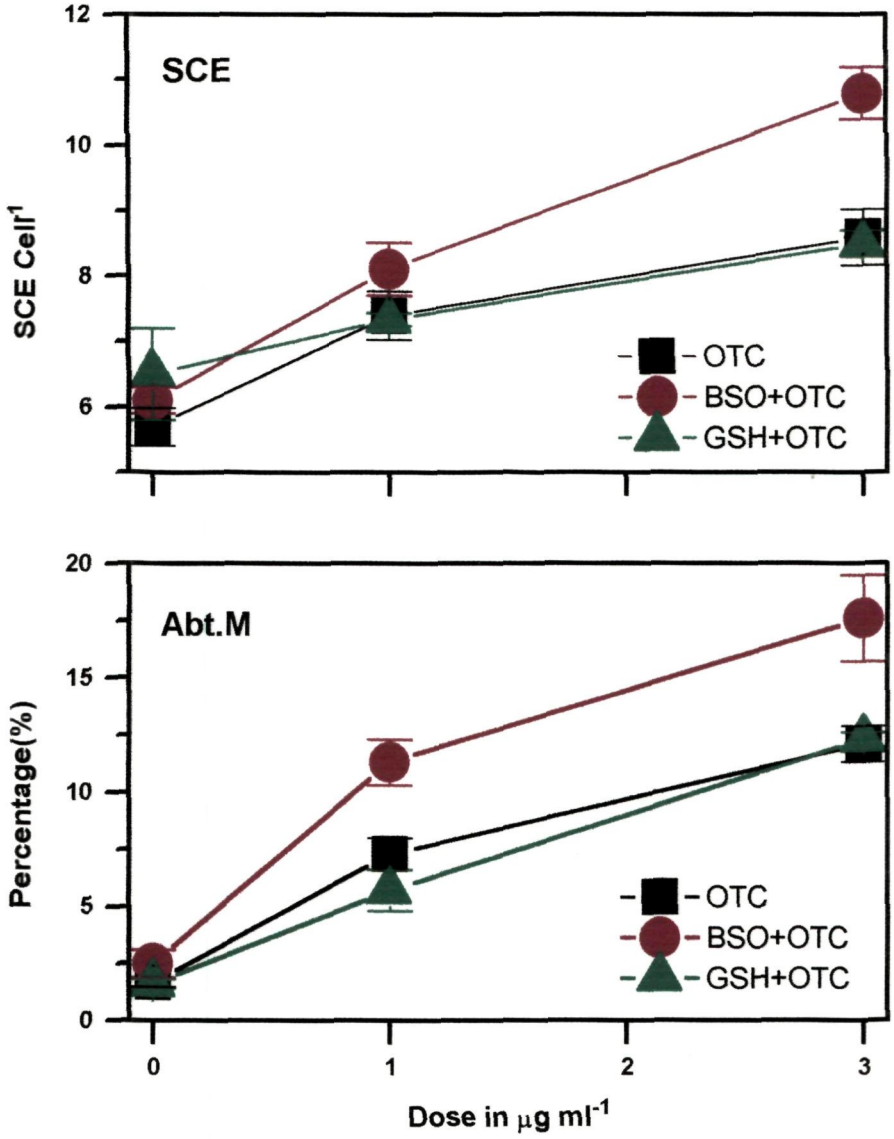


Fig. 2.7

Effect of OTC with or without BSO and GSH on the frequency of aberrant metaphases and SCEs in HPBLs. The plotted values are the mean ± SEM.

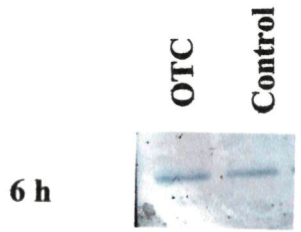


Fig. 2.8 Western blot analysis of TP53 (p53) in mouse bone marrow cells 6h after treated with OTC. 40 micrograms of protein was loaded in each lane.

Chapter 3.

Anti Tumor Activity of Et_2SnCl_2.L {L=N-[p-(2-pyridylmethylene) methylbenzenamine]}

Literature Review

One of the most outstanding developments in the field of metal compounds in medicine was Rosenberg's accidental discovery that platinum complexes possess anti-tumor activity (Rosenberg 1969). One of the first complexes discovered, cis-diamminedi-chloroplatinum(II) or cisplatin (Blunden 1985). However, drug resistance of tumor cells (Johnson *et al* 1993), toxicity (nephrotoxicity, ototoxicity, neurotoxicity) and side effects (emesis) frequently limit the clinical usefulness of cisplatin.

Organotin compounds show a spectrum of biological activities and have been extensively studied as fungicides, bactericides, acaricides and wood preservatives (Evans *et al* 1985; Saxena 1987). However, only scanty and scattered information is available on their activity against cancer, although some organotin compounds have yielded positive antineoplastic results against the P388 leukemia in mice than any other class of compound (Gielen 1986). These compounds have not receive much attention probably because the compounds tested have neither shown effectiveness against multiple types of cancer (Crowe 1984) nor produced test results which are as good as or better than those produced by the platinum compounds (Crowe 1980). Organotin compounds are known to interact with cell membranes and with proteins. The moieties R_nSn^{IV} (n= 2 and 3) bind to proteins and glycoproteins of cell membranes, as well as to cellular proteins: for example, Et_2Sn^{IV} to ATPase and hexokinase of trout, feline and human erythrocytes (Musmeci 1992). Feline and rat hemoglobin form complexes with R_nSn^{IV} and Me_2Sn^{IV} (Elliot *et al* 1979). Species such as $(Et_2Sn)_2$ (hemoglobin tetramer) are formed, characterized by high affinity between tin and hemoglobin (Musmeci 1992).

In 1973, Atushi *et al* reported the very high affinity of the tin to tumors. Although the first Organotin (IV) compound was tested for its anti-tumor activity in 1929, no systematic study was undertaken afterwards and as a result only over 1500 organotin compounds had been tested in various tumors system by the end of 1981 (Narayanan *et al* 1985). Diorganotin

compounds (Frust 1963) showed the most profound activity. In 1980, Crowe *et al* published the first detailed report on the anti-tumor activity of a series of diorganotin - dihalides and pseudohalide complexes $R_2SnX_2.2L$ (R = Me, Et, Pr, nBu or Ph; X = F, Cl, Br, I; 2L = bipyridyl, phenanthroline, 2-aminomethylpyridine; L = dimethylsulphoxide, pyridine etc.) A special feature of these complexes was that they were modeled on the active square-planar Pt(II) complexes which have cis halogen groups.

Much of the current interest dates back to the work of Brown in the early eighties. In her fundamental work, Brown noted that triphenyltin acetate exhibited anti-tumor activity in mice, whereas triphenyltin chloride was inactive (Brown 1972). She hypothesized that the degree of water solubility was an important factor in organotin anti-carcinogenecity. *In vitro*, antiproliferative and anti-tumor activity of two organotin (IV) carbohydrate compounds have been studied in different mouse tumor cell lines (Caruso *et al* 1993). They concluded that Sn-C bonded triphenyltin carbohydrates are less active than Ph_3SnCl. In mice bearing Ehrlich ascites tumor cells, Diphenyltin (IV) and diphenylantimony(III) derivatives of dithiophosphorous ligands inhibites tumor growth (Bara *et al* 1991).

The structure / activity relationship for diorganotin- dihalide complexes is that the Sn-N bond lengths appears to determine the anti-tumor activity (Crowe *et al* 1984). The more stable complexes exhibit lower activities. Those with an average Sn-N bond length larger than 2.39A° shows anti-tumor activity whereas those smaller than 2.39A° are inactive. This implies that a predissociation of the bidentate nitrogenous ligand might be a crucial step in the formation of tin-DNA complex (Gielen *et al* 1986).

It is widely believed that the effectiveness of many clinically useful anticancer drugs can be severely limited by the development of drug resistance. Tumor cells cultured *in vitro*, in particular those of human origin, were shown to contain extremely high levels of GSH (Biaglow *et al* 1983; Mitchell *et al* 1985). It has been shown that tumor cells made resistant to some anti-cancer drugs, e.g. melphalan, cisplatin and adriamycin, have increased

cellular GSH concentration (Green *et al* 1984; Hamilton *et al* 1985). An increased conjugation with glutathione has been proposed as a major mechanism in development of drug resistance towards alkylating agents. This has been attributed to the ability of GSH to compete with DNA for drug binding (Waxman 1993; Tsuchida, 1992). For these reasons much current interest has focused on techniques of reducing cellular levels of GSH prior to the treatment with anti-cancer agents. The ability of BSO to potentiate the anti-tumor activity of anticancer drugs has been demonstrated convincingly *in vitro* in human tumor cell lines for adriamycin, melphalan and cisplatin (Hamilton *et al* 1985) and *in vivo* for cyclophosphamide (Ono and Shrieve 1986) bleomycin and cisplatin (Tsutsui *et al* 1986). Therefore, an assessment of the influence of GSH on the anti-tumor activity of OTC is important since there is a growing list of new approaches to cancer therapy which do not rely only on the ability to block cell proliferation.

Materials and Methods

Anti Tumor Test

The Anti Tumor Activity of the new organotin compound $Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} was carried out in accordance with the U.S. National Cancer Institute standard protocols for primary screening.

The evaluation of this activity was established by computing the T/C value, which is the median survival time of the treated group if animals (T) divided by that of the control group (C).

The T/C ratio is given as a percentage. A compound is termed active if it has a T/C percentage $\geq$ 120% (Anon 1978).

Materials:

Dalton's Lymphoma Cells.(DL cells)

Described earlier in chapter 1.

Reagents:

$Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} (OTC).

A working solution of 1mg ml^{-1} was freshly prepared in 2% ethanol; the desired concentration was directly injected intraperitoneally in mice from the working solution.

DL-Buthionine-(S,R)-Sulfoximine (BSO, Sigma, USA)

Freshly prepared before used at a concentration of 1mg ml^{-1} in double distilled water.

Method:

The animals were divided into 4 groups of at least 10 animals each except one group, were only 5 animals were used and treated with cisplatin for the purpose of comparison.

Group 1: 1 x 10^6 Dalton's Lymphoma cells were inoculated and the survival time of each animal was recorded.

Group 2: 1 x 10^6 Dalton's Lymphoma cells were inoculated followed by OTC treatment on Day 1, 5, and day 9 at a dose of 15 mg kg^{-1}. The survival time of each animal was recorded in days.

Group 3: To this group, DL-Buthionine-(S,R)-Sulfoximine (BSO) at a dose of 50 mg kg^{-1} dissolved in double distilled water(1mg ml^{-1}) was injected 24 hours before OTC treatment.

Group 4: Cisplatin (1mg kg^{-1}) was treated to this in a similar manner as that of group 5. This was done only as a comparative study.

DL cell kinetics

Method:

OTC was treated on the 1^{st}, 5^{th}, and 9^{th} day after transplantation of tumor cells. On the 10^{th} day, the ascites fluid was collected washed in RPMI 1640 medium and these samples were cultured in RPMI 1640 medium supplemented with 10% heat inactivated new born calf serum and a growth factor Insulin-Transferrin Selenium A supplement (Gibco,USA), 10µl ml^{-1} medium and incubated at 37°C. To obtained differential sister chromatid staining, 6µg ml^{-1} BudR was added to the cultures. Cells were harvested at

48h, and colcemid (0.01μg ml^{-1}) was added 8h prior to termination of cultures.

Fixation of cells, metaphase preparation and differential staining procedure were similar to that of *in vitro* studies described earlier.

Results

Anti-tumor activity

The Treated / Control values are given in Table 3.1. Data indicate the very high activity of OTC on DL cells. The T/C value was 146% when OTC was treated on the 1^{st}, 5^{th} and 9^{th} day after transplantation. However, this T/C value was much below that of cisplatin treatment. The dose of cisplatin (1mg kg^{-1}) is equivalent to 15 mg kg^{-1}) OTC, since both showing similarity in the induction of chromosomal aberrations in mouse bone marrow cells (Table 3.2). The observed T/C value of the OTC further improved considerably when BSO was treated on the 4^{th},and 8^{th} day (24h before OTC treatment) after transplantation. BSO alone showed an insignificant T/C value (Fig.3.1).

DL- cell kinetics

Table 3.3 shows the frequency of M1% and M2% cells. The percentage of M1 was higher in OTC treated sample indicating a delay in cell cycle progression. The range of M1 was 71 to 82% and 86 to 100% in untreated and OTC-treated samples respectively. The AGT was significantly increased in the groups treated with OTC.

Discussion

Over the last few years, the use of organotin compounds as a pharmaceutical products had received the much-needed attention among many organo-metallic chemists and biologists. It has been reported in the literature that various organotin material retard both onset and growth of cancer in mice (Cardarelli *et al* 1984; Carrara *et al* 1989; Geilen *et al* 1995; Geilen *et al* 1996). The results indicate that the present OTC inhibits cell proliferation and shows anti-tumor potentialities, which is influenced by the endogenous GSH-level. Treatment with BSO produces a rapid decrease in the GSH levels of the various tissues (Lee *et al* 1987). In DL-cells the total GSH estimated indicates that 4 and 24h incubation with BSO (50 mg kg^{-1}) could deplete 75% and 47% of endogenous-GSH level respectively with respect to control. Such a huge level of depletion could probably be the reason for the inability of the tumor bearing-mice to survive when OTC was treated 4h post BSO treatment. Interestingly, it has been observed in this study that OTC alone increased the endogenous GSH significantly in DL-cells, which could be associated with the cells resistance to OTC. In the present study it is possible that OTC treatment after 4 and 10h of BSO treatment could not increase endogenous GSH due to impairment of GSH-synthesis by single BSO treatment and therefore the toxicity of OTC increased which could be the reason for increased mortality when OTC was given after BSO-treatment. Keeping this in mind, OTC was treated 24h after BSO treatment in the anti-tumor study. Studies on several cell lines showed that cellular glutathione levels are greatly increased. This may be because cells synthesize GSH rapidly in response to stress. High level of GSH makes many tumor cells resistance to chemo-and radio-therapy (Meister 1994). GSH may also have a role in modulating the mode of cell death following toxic injury (Fernandes *et al* 1994). The observation that GSH depleted leukemia cells undergo necrosis when exposed to melphalan, while non-GSH depleted cells undergo apoptosis supports this hypothesis (Fernandes

et al 1994), in addition GSH is an antioxidant and a scavenger of free radicals (Deleve 1991).

With regards to the mode of action of anti-tumor active organotins, no explanations are presently available from results obtained with tumor cells. On the basis of studies of Crowe *et al* it seems unlikely that organotins compounds will interact with DNA by cross-linking Sn with suitable oriented nitrogen bases as appears to explain the anti-tumor activity of cisplatin and its analogs. But, in the present study, the compound under investigation has a bidentate ligand L {L=N-[p-(2-pyridylmethylene) methylbenzenamine]}, which ensure that the resulting octahedral complex possessed cis-halogens, the ligand L makes this new complex structurally similar to cisplatin.

Since its discovery by Rosenberg, a wealth of information has been published on the interactions of cisplatin with nucleotides and DNA (Sherman *et al* 1987; Reedijk *et al* 1987). These studies have supported the theory that cisplatin enters healthy as well as tumor cells and then reacts specifically with intracellular DNA, thus inhibiting proliferation. This theory also appears to be true in the case of the present OTC. It was observed that there was a delay in the cell cycle kinetics of DL-cells. The M1% increased significantly in OTC-treated samples compared to untreated ones. The precise molecular nature of the different adducts formed from cisplatin and DNA has been extensively studied (Sherman *et al* 1987; Reedijk *et al* 1987). The complexes are believed to lose their chloride ligands and the metal subsequently co-ordinates with suitably oriented nitrogenous bases of DNA (Prestayko1980). Cisplatin favors binding to the N7 atom of the DNA base guanine which can result in inter- or intra- strand cross-linking of adjacent or opposing guanine moities as well as cross-links between guanine and a protein molecule (Kratz 1998). The original concept seems to hold that N7 position is important for chemotherapy (Brookes 1990). Since the tin complexes were structurally similar to those of platinum, it is expected that their mode of action would also be similar.

As far as the mechanism of action of the other organotin compounds is concerned, only tentative hypotheses can be formulated. Among all, it appears that they might act through the thymus and likely the lymphatic system (Cordarelli *et al* 1984) by disturbing cellular glucose metabolism, energetic and macromolecular synthesis (Penninks *et al* 1990).
Since soluble organotins of varying types, introduced orally or by injection in mice, are concentrated in the thymus gland and tin content in tumors is lower compared to normal tissue, it is also hypothesized that these compounds are converted to anticarcinogenic organotin in the thymus, probably in a steroid form that kills tumor cells or prevents their proliferation (Barbieri 2000).

Whether all or any of these mechanisms contribute to the observed anti-tumor activity of OTC remains to be determined. The observations that OTC possesses cytotoxic activity and anti-tumor potential against Dalton's Lymphoma cells development in experimental animals have significant implications for the future development and management of the drug. The purpose of cancer treatment in experimental animals and in humans is to reduce the viable tumor cell population to a number below which the cells surviving drug treatment are not to re-establish the grossly evident and ultimately fatal disease. Cure is the desired final goal (Schabel 1978).

Table 3.1 Anti-tumor activity of OTC (15 mg kg^{-1}) towards Dalton's Lymphoma.

Exp. Condition	No. of animals	BSO treatment (days)	OTC treatment (days)	Median survival time(days)	Range survival time(days)	T/C %
Untreated	10	-	-	13	10-16	-
BSO	02	4^{th} ,8^{th}	-	13	11-13	100
OTC	11	-	1^{st},5^{th},9^{th}	19	14-28	**146**
BSO+OTC	10	4^{th}, 8^{th} *	1^{st},5^{th},9^{th}	25	19-31	**192**
Cisplatin 1mg kg^{-1}	05	-	1^{st},5^{th},9^{th}	35	22-39	269

* 24 hours before OTC treatment.

Table3.2 Effect of cisplatin and OTC on chromosomal aberration induction in mouse bone marrow cells

Cispatin (1mg kg^{-1})		**OTC (15mg kg^{-1})**	
TM	Ab. M(%)	TM	Ab. M(%)
116	1.75	128	1.56
106	2.80	103	2.91
		201	3.98

TM; Total Metaphases. Ab.M; Aberrant metaphases.

Table 3.3. Effect of OTC (15 mg kg^{-1}) on cell cycle kinetics of Dalton's lymphoma cells *in vitro* fixed at 48 hours.

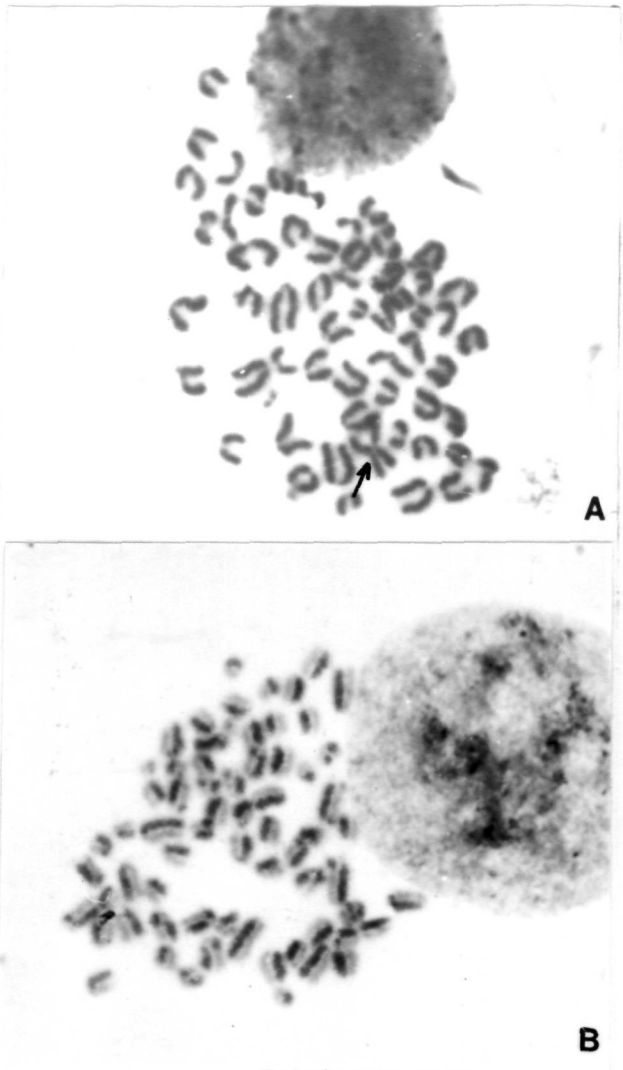
Expt. Condition	TM	M1%	M2%	Mean M1% ± SEM	AGT (H)	Mean AGT ± SEM
Untreated	099	079	21		40	
	085	082	18		40	
	090	076	24		40	
	106	071	29	77 ± 2.34	37	39.25 ± 0.75
OTC	078	086	14		44	
	079	094	06		46	
	091	100	00		48	
	075	100	00	95 ± 3.31#	48	46.50 ± 0.95

TM; Total Metaphases. M1; 1st cycle metaphases.
# $p<0.05$ 2x2 contingency χ^2 – Test.

Fig 3.1: Microphotographs showing Hoechst-Sunlight-Giemsa staining pattern of chromosomes in Dalton's lymphoma cells grown in the presence of BudR.

A. One division cycle showing marker chromosome (→).

B. Two division cycle.


A
B

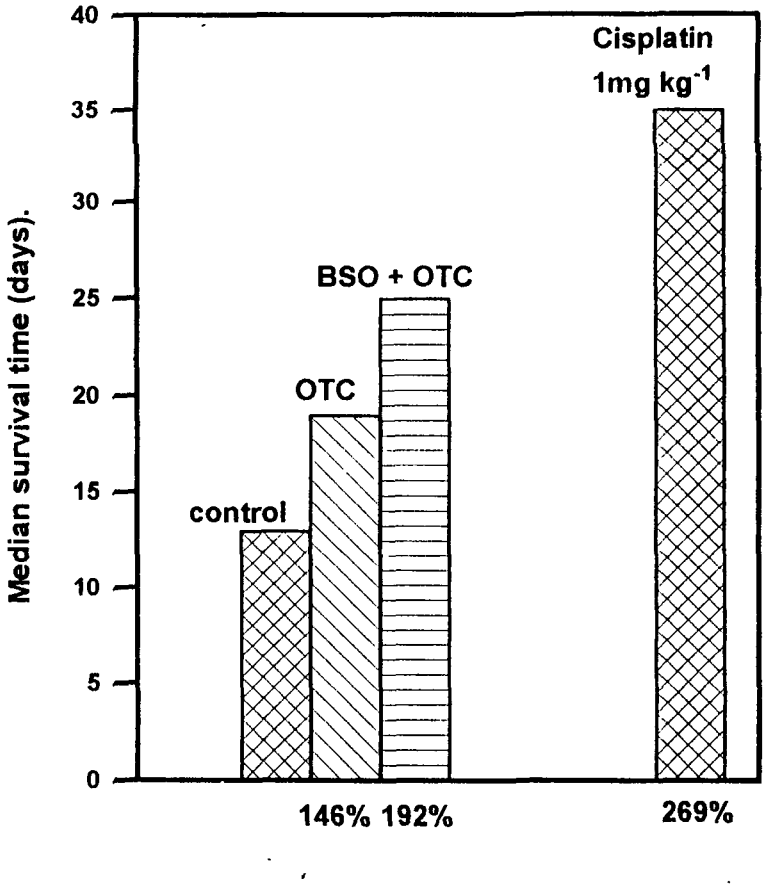


Fig. 3.2

Antitumor activity of OTC and cisplatin against Dalton's lymphoma transplanted in mice. The T/C value is given in percentage.

Chapter 4.

Does $Et_2SnCl_2.L$ {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} Induce cell death?

Literature review

The evidences gathered so far, in this study indicates that this new compound shows genotoxic properties as well as anti-tumor potentialities. Endogenous GSH level also have proved to be an important factor in both cases. In continuation with the results obtained in the previous chapters, the analyses of cell death and the pattern of such cell death has been performed in order to determine the mechanistic aspects of the anti-tumor potentiality of the present compound.

Apoptosis is well recognized as a distinct pathological mechanism in tumors responding to anticancer therapies (Eastman 1990; Dive and Hickman 1991). Hence, it seems plausible to also determine if the present OTC induces cell death. Apoptosis is a distinct form of cell death that involves an intrinsic normal cell death program. It is encoded intrinsically by a "suicide" genetic program that is triggered when cells are exposed to certain intracellular or extra-cellular stimuli (Wyllie 1980; Arends and Wyllie 1991). Normal cell death was first observed during amphibian metamorphosis in 1842. It was soon found in many developing tissues in both invertebrates and vertebrates (Jacobson 1997). In 1972 Kerr and his colleagues introduced the terminology "apoptosis" to provide a distinction between this cell death that occurs in both animal development and tissue homeostasis and on the other hand pathological cell death (largely known as necrosis) that occurs in acute lesions from trauma and ischemia (Kerr *et al* 1972). Apoptosis is distinct from necrosis in several ways. Unlike necrosis, apoptosis is generally not associated with inflammation. Apoptotic cells are somehow recognized and engulfed by neighboring cells and macrophages, leaving the tissue with minimal damage. Gene transcription and protein synthesis are often required for cells undergoing apoptosis but not for necrosis, indicating an active role for newly synthesized gene products. In addition, apoptosis is usually associated with the activation of specific endonuclease that cleaved DNA between nucleosomes, leading to

chromosomal degradation. It is now widely recognized that apoptosis is important for normal tissue turnover, because it allows for the precise regulation of cell numbers. It is now clear that apoptosis also serves as a defense mechanism, eliminating potentially dangerous cells, such as virus infected cells and cells exposed to toxins or other adverse environmental conditions (Thompson *et al* 1995; Meyn *et al* 1996).

Genetic evidence also indicates that activation of apoptosis is an important event for tumor suppression. Over half of human cancers have mutations in p53, a gene that is important for induction of apoptosis under genotoxic stress (Hollstein *et al* 1991; Levine *et al* 1991). If cell proliferation and cell death are thought of as a ratio, then tumor mass will increase if proliferation increases or death decreases. This new understanding is beginning to open up new approach to cancer therapy, focusing on devising mechanisms to stimulate selective apoptotic death of cancer cells and consequently reduce tumor mass (Hickman 1992).

Materials and Methods

Trypan Blue Viability Test

The fact that viable cells do not take up certain dye whereas dead cells do was utilized to determine the possible mode of action of OTC. The cell viability test was determined after different timings of the drug treatment.

Reagents:

1. Preparation of Trypan Blue stain:

(a) Trypan blue 0.4g (Hi Media Laboratories Pvt. Ltd. India)
(b) Sodium Chloride 0.81g (NaCl; Merck India Ltd.)
(c) Dipotassium Hydrogen Phosphate 0.06g (K_2HPO_4; Merck India Ltd.)
(d) Methyl - ρ - hydroxybenzoate 0.05g (Loba Chemie Pvt. Ltd.)

All the above reagents were mixed together along with 95 ml double distilled water heated until it is completely dissolved.
After cooling the pH is adjusted to 7.2 to 7.3 with 1N NaOH.

2. Hanks' balance salt solution. (HBSS)

3. Culture media (RPMI 1640: Hyclone, USA.)

Methods:

DL cells viability was measured after 7 days of intraperitoneal transplantation by counting cells stained in Trypan blue after 2,4,17,and24 hours of OTC treatment. When BSO was used, it was treated 24h before OTC treatment. The cell suspension was centrifuged in media at 5000 rpm for 5 minutes, the supernatant was discarded and the cells were resuspended in 1.0 ml HBSS, Ca^{2+} and Mg^{2+}- free.
1 ml of Trypan blue dye was added and after 4 - 5 minutes the viability of the cells was checked under a microscope. Dead cells take up the stain while live ones do not (Fig 4.1). At least 1000 cells were considered and the result was expressed in percentage.

Detection of Apoptosis

Reagents:

1. Methanol / Acetic acid (Merck India Ltd.) at a ratio of 20:1.

2. Haematoxylin and Eosin Stain (s.d's fine chemicals India Ltd.)

3. Phosphate buffer saline (PBS tablets, pH 7.4)

Methods:

For light microscopic observation, the method of Vral et al (1987) was followed. DL cells were washed in PBS and fixed in suspension using methanol / glacial acetic acid (20 / 1). After 30 min fixation, the cells were centrifuged, concentrated in a small amount of fixative and dropped gently onto slides with a Pasteur pipette. After air-drying, slides were stained with haematoxylin and eosin during 40 min at 40°C. 1000 cells were scored from each sample. DL cells with a pyknotic nucleus were scored as apoptotic; cells containing a normal nucleus with dispersed heterochromatin were scored as viable. (Fig. 4.1)

Results

Trypan Blue Exclusion Test

The percentage of Dead cells was observed in the Trypan Blue dye Exclusion Test after OTC treatment with and without BSO is presented in Table 4.1. In untreated samples 5.5% dead cells was observed. The number of dead cells increased linearly in samples collected 2,4,17 and 24h after

OTC treatment. This increased further at 17 and 24h samples when BSO was treated 24h before OTC treatment. It is worth mentioning that BSO alone-induced cell death and therefore it is necessary to consider that BSO per se induced an increase of 4.9% with respect to untreated samples. This means that considering the additive increase of BSO + OTC induced an increase of 4.9+11.9=16.8 dead cells per 100 cells at 17h of sampling time which was compared with the observed increase of 21.6% indicating 29% increment. The data in Table 4.1 also shows the percent increment of dead cells in combined treatment. This enhancement was clear at 17 and 24h samples whereas 2 and 4h samplings, BSO could not increase the number of dead cells induced by OTC.

Induction of Apoptosis

In order to evaluate the apoptotic induction ability of OTC a simple and crude method by light microscopy has been made and the data presented in Table 4.2. Lymphoma cells with pyknotic nucleus were scored as apoptotic and cells containing a normal nucleus with dispersed heterochromatin were scored as viable. Light microscopy scoring of apoptotic cells were performed in untreated, BSO, OTC and BSO+OTC treated samples. The number of OTC treated apoptotic cells was obtained by subtraction of the number of cells scored as apoptotic in the control samples from the total number of apoptotic cells scored in the OTC treated samples. Both OTC alone and in combination with BSO showed significant increase in the apoptotic induction after 24h of OTC treatment with respect to their respective control. Since BSO alone showed slight increase in percentage of apoptotic cell death with respect to control, an additive effect of both BSO and OTC when both were combined were also considered. Data presented in Table 4.2 indicate that expected increase in combined samples is 4.69% but the observed increase was 7.72%, which indicates a 64% increment.

Discussion

Apoptosis is well-recognized pathological mechanism in tumors responding to anticancer therapies (Eastman 1990; Dive and Hickman 1991). Present observed increase in survivality of mice described in the previous chapter could be due to both inhibiting cell proliferation and subsequently killing the cells by the OTC. The OTC induces delay in cell kinetics in mouse bone marrow cells and such inhibition in cell proliferation has been reported for dibutyltin chloride (DBT) and trimethyltin chloride (TMT) in normal isolated B cells (De Santiago *et al* 1999) and in DNA synthesis in mouse spleen cells (Al-Imara *et al* 1993). Evidences are also there for several organotin compounds, which exhibited antiproliferative activity against tumor cell lines and retard both the onset and growth of cancer in mice (Carrara *et al* 1989; Gielen *et al* 1996). Therefore, it could be inferred that the OTC increased the life span of tumor bearing mouse by inhibiting cell proliferation. However, it has been reported that organotin compounds increase cytosolic Ca^{2+}, alter functionality and induce apoptosis in rat thymocytes (Peiters *et al* 1994; Gennari *et al* 1997) and in vitro exposure diminish the viability of mouse spleen cells and B cell hybridoma (Al-Imara *et al* 1993; Thompson *et al* 1996). Therefore, we have pursued further study in order to see whether the OTC also induces cell death or not. Trypan-blue dye exclusion assay indicate that there was an increase in cell death after 17 to 24h of OTC-treatment and this was increased further when BSO was treated 24h before OTC-treatment. Diphenyltin (IV) has been tested *in vitro* and *in vivo* against Ehrlich ascites tumor and exhibited inhibitory effects on cell proliferation, viability and protein synthesis (Bara *et al* 1991). It has been shown that both DBT and TMT induced apoptosis, cell death and decreased proliferation in stimulated 72h cultured B-cells (De Santiago 1999). Stridh *et al* (1999) demonstrated that tributyltin and triphenyltin could kill target cells by triggering apoptosis in human Hut-78 and Jurkat T-lymphocyte cell-lines by increasing the caspase activity. Therefore,

in order to know whether, the present observed cell death could be partly due to apoptotic cell death or not we have analyzed apoptotic cells under light microscope. The results indicate that 24h after OTC-treatment there was significant increase in apoptotic cell death and interestingly such increase was more when BSO was treated 24h before OTC-treatment. It was demonstrated earlier that GSH might have a role in modulating the mode of cell death following toxic injury (Fernandes *et al* 1994). The role of GSH in modulating the cytotoxicity of platinum complexes (Pendyala *et al* 1997) and Gamma radiation (Chattopadhyay 1999) by affecting DNA-repair, apoptosis and free radical scavenging has also been demonstrated. Markovie *et al* (1997) also showed that depletion of cellular thiol levels by exogenous thiol-modifying agents susceptibility to radiation-induced apoptosis was restored in the LY-ar cell line. Therefore, present data indicate that the OTC could induce apoptosis, and such induction was more in GSH-depleted condition. This could also explain why OTC treatment in BSO-treated mice showed less delay in cell proliferation since the number of first cycle cells could probably decrease due to apoptotic death (Data shown in chapter 2).

It is very important to know how apoptosis is triggered because this maybe necessary for successful treatment. Do genes such as E1A, p53, myc etc that induce apoptosis do so through the same biochemical pathways by which they regulate the cell cycle? It is attractive to think so, but few mechanistic details are known. In order for cell growth and development to occur, the cell cycle arrest and apoptotic functions of p53 and other family members need to be tightly regulated and activated only when necessary and appropriate. It has now become clear the activity of p53 is regulated primarily through control of protein stability (Kubbutat and Vousden 1998).

It may not be incorrect to feign that cell cycle delay and apoptosis observed in this study may occur in a p53-dependent manner. p53–induced apoptosis is induced by DNA-damage, hypoxia or chemotherapy (Levine 1997). In most cases, p53 induced apoptosis appears to be independent of

its transcriptional function because it occurs in the presence of protein synthesis inhibitors. Protein-protein interaction between p53 and factors involved in the DNA repair mechanism can account for additional ways by which p53 induces apoptosis without transcriptional activation (Wang 1995). The extents of DNA-damage and p53 protein levels are factors that contribute to making the choice between life and death. It may be that during p53-induced cell cycle arrest, the cell attempts to repair damage, but if the damage is too extensive to be repaired, the cell is then committed to die (Levine 1997).

The tremendous increase in our understanding of apoptosis and its relevance to cancer has produced both good news and bad. The good news is that striking connections have been made among p53, apoptosis, oncogenesis and treatment outcome. The bad news is that p53 disruption is extremely common in human cancers. (Fisher 1994) A major mode of resistance to anti-tumor treatments may be insensitivity to apoptosis induction. These conclusions stem from studies of select cell types, oncogenes and apoptosis triggers. Their application to most human cancers, if verified, stands to revolutionize our approach to cancer therapy.

Table 4.1. Number of dead cells caused by OTC (15mg kg^{-1}) alone or with BSO (50 mg kg^{-1}) in Dalton's Lymphoma cells.

Exp. condition	No. of animals (dead)	Cells collected after(h)	Mean Dead cells %	Range Dead cells %	Dead cells Increment Observed	Expected	Increment %	
Untreated	10(0)	-	05.5 ± 0.33	04.1 - 06.9	-	-		
	05(0)	2	09.8 ± 0.70	09.0 -10.7	04.3	-		
	05(1)	4	11.4 ± 1.30	09.2 -15.2	05.9	-		
	10(3)	17	17.4^{+} ± 3.18	07.1 -28.3	11.9	-		
	10(3)	24	22.4^{+} ± 2.64	10.2 -31.8	16.9	-		
BSO	10(0)	24	10.4 ± 0.62	07.7 -13.5	04.9	-		
BSO + OTC	05(1)	2	11.1 ± 1.06	09.1 -14.1	05.6	09.1	Not increased	
	05(1)	4	11.5 ± 1.19	09.1 -14.9	06.1	10.8	Not increased	
	10(4)	17	27.2^{+} ± 1.98	20.8 -34.0	21.7	16.8	29%	
	10(4)	24	30.6$^{\$}$ ± 1.73	24.2 -36.0	25.1	31.8	15%	

$^{+}$ $p<0.001$ 2x2 contingency χ^2 - Test; compared with control value.
$^{\$}$ $p<0.05$ 2x2 contingency χ^2 - Test; compared with OTC (24h) only.

Table 4.2. Percentage of Apoptotic cells observed after treatment with OTC (15mg kg^{-1}) alone or in combination with BSO (50mg kg^{-1}) in Dalton's Lymphoma Cells.

Expt. condition	Fixation time (h)	Apoptotic cells %	Mean ± SEM	Observed increase	Expected increase	Increment (%)
Untreated	-	02.90				
		02.50				
		02.02				
		02.80				
		02.70	02.58 ± 0.34	-	-	
BSO	24	03.43				
		03.27				
		03.37				
		03.75	03.45 ± 0.27	0.87	-	
OTC	24	06.10				
		07.50				
		05.80				
		06.50				
		06.19	06.40* ± 0.65	3.82	-	
BSO + OTC	24	11.50				
		11.60				
		08.90				
		09.10	10.30$ ± 1.47	7.72	4.69	64

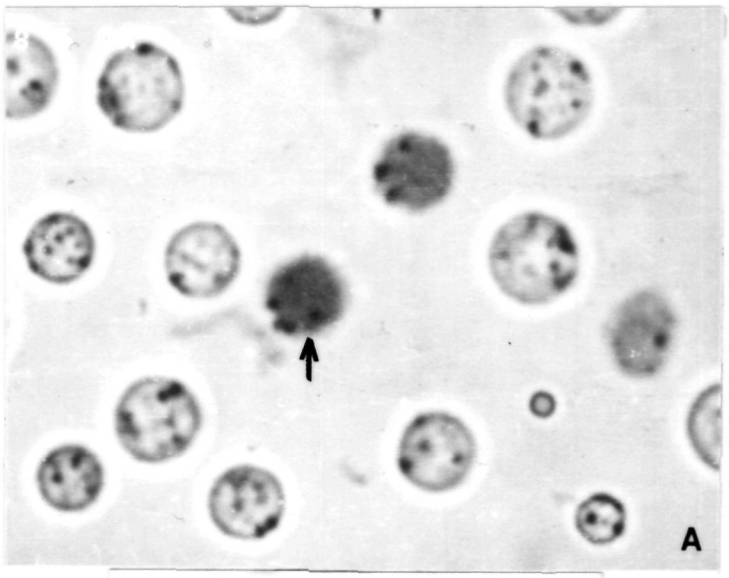
In each sample at least 1000 cells were considered.

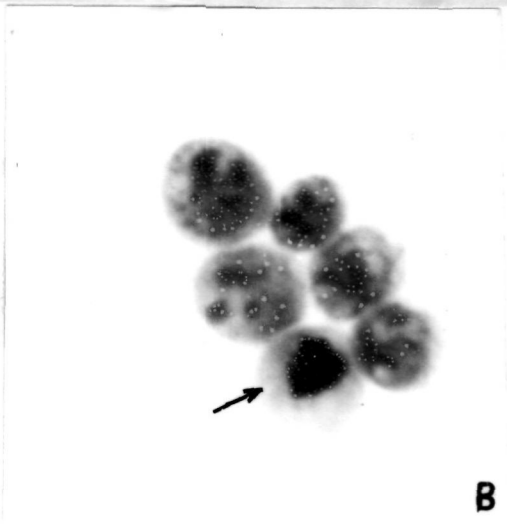
*$p<0.001$ 2x2 contingency χ^2 - Test; compared with their control value.
$^{\$}p<0.01$ 2x2 contingency χ^2 - Test; compared with their control value.

Fig.4.1: Microphotographs showing cell death in OTC-treated Dalton's lymphoma cells.

A. Trypan Blue Dye Exclusion Test (→) showing non-viable cell.

B. Apoptotic cell (→) showing pyknotic nuclei after fixation with methanol/acetic acid.


A


B

Summary

Organotin compounds are organometallic compounds having varying degrees of toxicological properties They are known to interact with cell membranes and with proteins. The toxic action of many organotin compounds has been ascribed to their tendency to combine with coenzymes and enzymes possessing dithiol group. Our current knowledge on cellular effects of organotins is mainly restricted to studies with cells of the immune system because of the predominant immunotoxic effects of various organotin homologues. Organotins have also been known to increase cytosolic Ca^{2+}, alter functionality and induce apoptosis in rat thymocytes. Several of these compounds showed positive antineoplastic effect against the P388 leukemia in mice. But, organotins have not received much attention as the platinum compounds had, probably because those tested have neither shown effectiveness against multiple types of cancers nor produced test results which are as good or better than those produced by the platinum compounds. There are also loopholes in available literatures that make it difficult to assess the relative mutagenic potential of these compounds. A structural correlation with biological activity for diorganotin complexes has shown that active species are associated with complexes having Sn-N bond length longer than 2.39 A° which in turn would determine the formation of tin-DNA complex. The present new compound Et_2SnCl_2 L {L=N-[p-(2-pyridylmethylene) methylbenzenamine]} (OTC) has an Sn-N bond length of 2.46A° which is larger than 2.39 A° and is expected to facilitate the formation of tin-DNA complex.

Depending on their structure, most DNA adducts either slow or block DNA replication. This event may itself lead to arrested cell division and / or DNA damage (Weinberg 1989). Given the fact that, the present tin compound is believed to lose its ligand, facilitating the formation of tin-DNA adduct, it seems logical to conclude that the drug directly attacks the DNA and producing its genotoxic potentialities

In this study, OTC has been subjected to investigations such as the antiproliferative and genotoxic activity against mammalian cells both in vivo

and in vitro in relation to the cellular GSH-level since it play an important role in cellular defense mechanisms.

The levels of GSH were estimated in both the normal as well as the tumor systems used in the present investigation. This was carried out with and without BSO treatment and also after OTC treatment following the method of Akerboom and Sies (1981). The activity of GSTs was also determined post OTC treatment by the method of Habig *et al* (1974).

Genotoxic studies were carried out *in vivo* and *in vitro* and the endpoints determined were cell cycle kinetics, chromosomal aberrations and sister chromatid exchanges. In *in vivo* system, OTC 15mgkg^{-1} was injected intraperitonially into male Swiss albino mice aged 2-3 months weighing 25-30g, 30 mins after subcutaneous implantation of BrdU tablets. BSO 200 mg kg^{-1} injected ip 10 hrs prior to OTC treatment. When GSH was used, it was added 30 mins after the implantation of the BrdU tablets and OTC was treated 30 mins after GSH treatment. For the *in vitro* system, heparinized peripheral blood from healthy male donors was used immediately after venipuncture. OTC (1and 3 μgml^{-1}) was added to the blood for 2 hrs. BSO (5 mM) was added into 1ml aliquot of whole blood for 3hrs and then OTC was added. In the sample where GSH was used, it was added 30 mins before OTC treatment. The samples were incubated at 37°C with medium RPMI1640 supplemented with 10% heat inactivated serum following PHA stimulation. For the differential staining, 5μgml^{-1} BrdU was added to the cells at the initiation of the cultures. Cells were harvested at 72 hrs adding colcemid 3 hrs prior to the harvesting time.

The antitumor activity was measured in Dalton's lymphoma cells, which were maintained by serial intraperitonial transplantation in 3 months old Swiss albino mice using an inoculums size of 10^6 cells per mouse. This was carried out in accordance with the US National Cancer Institute standard protocol for primary screening. Some preliminary work was also done to determine the mechanism of action of OTC on DL cells. For this, the DL cell cycle kinetics study was carried out *in vitro*, and cell death was determined

by the Trypan Blue dye exclusion test. Apoptotic studies were also carried out using light microscopy.

From the present study, the following conclusions can be drawn:

1. OTC-treatment alone increases the endogenous GSH level in both the normal and tumor systems. The activity of glutathione-S-transferases was also enhanced post OTC-treatment.
2. OTC induces genotoxic effects in normal as well as tumor system.
    - Induces delay in cell-cycle kinetics in mouse BMCs, HPBLs and DL cells
    - Significant level of CAs in HPBLs, but not in mouse BMCs
    - Significant level of SCEs induction in both *in vivo* and *in vitro* systems.
    - Trypan Blue dye exclusion assay indicates that OTC induces cell death.
    - Triggers apoptotic cell death in DL cells.
    - The genotoxic effect is more pronounced in presence of a GSH-depleting agent in all systems.
3. OTC increases the survivality of DL-bearing mice and this improved further when BSO is treated before OTC-treatment.
4. From the preliminary studies, it appears that cell-cycle delay and apoptosis induced by OTC is dependent on the p53 protein.

There are two strategies in the development of a new anticancer drug, first is to screen the potential agents for cytotoxic properties and then to test these agents for their effectiveness against tumor cells. Again, it is mandatory to screen new drugs for their mutagenic effect before they are released. Keeping these facts in mind, the present investigation was undertaken and the objectives achieved

References

Abe S and Sasaki M. Chromosome aberration and sister chromatid exchanges in Chinese hamster cells exposed to various chemicals. J. Natl. Cancer Inst. 58: 1635-1640. 1977.

Adiar FE and Bagg HJ. Experimental and clinical studies on the treatment of cancer by dichloroethyl sulfide (mustard gas). Ann. Surg. 93: 190. 1931.

Agarwal S, Mathur M, Srivastava A, Ralhan R. MDM2/p53 expression in oral pre-malignant and malignant lesions: potential prognostic implication. Europ.J Cancer B Oral Oncol. 1998

Akerboom TPM and Sies H. Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples. Methods in enzymology 77: 373-382. 1981

Al-Imara L, Salaman MR, Sljivic VS, Poller RC. Inhibition of mouse spleen cell activity by organotin compounds: effect of attachment to a maltose residue to the organotin group. Int J of Immunophar. 15: 287-291, 1993.

Anderson ME, Naganuma A, and Meister A. Protection against cisplatin toxicity by administration of glutathione ester. FASEB J., 4: 3251-3255. 1990.

Andrews PA, Murphy MP, Howell SB. Differential sensitization of human ovarian carcinoma and mouse L1210 cells to cisplatin and melphalan.

Anon. Guidelines for the testing of Chemicals for Mutagenicity, Report on health and social subjects, No. 35, Department of Health, HMSO, London, 1989.

Anon 'Screening Data Summary Interpretation' U S National Cancer Institute Instruction 14 1978.

Anttila S, Luostarinen L, Hirvonen A. *et al.* Pulmonary expression of glutathione S- transferase M3 in lung cancer patients: association with GSTM1 polymorphism, smoking, and asbestos. Cncer Res. 55(15) 3305-3390 1995

Arcamone FM, Animati F. Barbieri B, Configliacchi E, D'Alessio, Geroni C Giuliani FC, Lazzari E, Menozzi M, Mongelli N, Penco S. and Verini MA Synthesis, DNA binding properties and anti-tumor activity of novel distamycin derivatives J Med Chem. 32 774-778. 1989.

Arends MJ and Wyllie AH Apoptosis mechanisms and role in pathology Int Rev of Expt Pathology, 32 223-254 1991

Armstrong DK, Gordon GB, Hilton J, Streeper RT, Colvin OM, Davidson NE Hepsulfam sensitivity in human breast cancer cell lines: The role of glutathione and glutathione S- transferase in resistance: Cancer Res 52:1416-1421. 1992

Ashby J and Paton D The influence of chemical structure on the extent and sites of carcinogenesis for 522 rodent carcinogens and 55 different human carcinogens exposures. Mutation Res. 286: 3-74. 1993.

Ashcroft M and Vousden KH. Regulation of p53 stability. Oncogene18: 7637-7643 1999

Atassi G Rev Silicon, Germanium, Tin, Lead Compounds 8: 219, 1985.

Attahiru US, Iyaniwura TT, Adaudi AO, Bonire JJ. Acute toxicity studies of tri-n-butyltin and triphenyltin acetate in rats. Vet. Hum. Toxicol. 33: 554-556. 1991.

Atushi A, Hisada, K, and Ando I. Radioisotopes, 22: 7. 1973.

Bara A, Socaciu C, Silvestru C, Haiduc I. Antitumour organomettallics. I.activity of some diphenyltin(IV) and diphenylantimony(III) derivatives on in vitro and in vivo Ehrlich ascites tomour. Anticancer Res 11: 1651-5, 1991.

Barbieri F, Viale M, Sparatore F, Favre A, Cagnoli M, Bruzzo C, Novelli F, and Alama A. Cytotoxicity *in vitro* and Preliminary Antitumor Activity *in vivo* of a novel Organotin compound. Anticancer Research 20: 977-980. 2000.

Basu Baul S, Basu Baul TS, Rivarola E, Dakternieks D, Tiekink ERT, Syng-ai C, Chatterjee A: Synthesis and characterization of diorganotin(IV) complexes of N-(2-Pyridylmethylene)-arylamines and Mutagenecity testing of $Et_2SnCl_2L^4$ [L^4=N-(2-Pyridylmethylene)-4-toluidine]. Appl Organomettallic Chem 12: 503-513, 1998.

Batist G, Tulple A, Sinha BK *et al* Overexpression of a novel anionic glutathione transferase in multidrug-resistance human breast cancer cells. J. Biol. Chem. 261: 155444-9. 1986.

Beck B and Obe G. The human leukocytes test systems. IV) The use of sister chromatid exchanges as a possible indicator for mutagenic activities. Humangenetik 29: 127-134, 1975.

Benditt EP and Benditt JM. Evidence for a monoclonal origin of human atherosclerotic plaques. Proc.Natl. Acad.Sci. USA. 70: 1773. 1973.

Biaglow JE, Tutle SW. The role of glutathione and associated enzymes in the cellular response to radiation, peroxide and hydroperoxides. In : Drug Resistance in oncology, Teicher BA (ed) 309-337. Marcel Dekker : New York. 1993

Biedler JL Genetic aspects of multidrug resistance (review). Cancer 70: 1799-1809 1992

Blair SL, Heerdt P, Sachar S, Ablhoda A, Hochwald S, Cheng H, Burt M. Glutathione metabolism in patients with non small cell lung cancers. Cancer Res 57: 152-155. 1997.

Blunden SJ, Cusack PA and Hill R. The Industrial uses of Tin Chemicals. Royal society of Chemistry : London 1985.

Bollo E, *et al.* Triphenyltin acetate toxicity : a biochemical and ultrastructural study on mouse thymocytes. Human and Experimental Toxicology. 15: 219-225. 1996

Bolton MG, Colvin OM, and Hilton J. Specificity of isoenzymes of the hepatic glutathione S-trasferases for the conjugation of glutathione with L-phenylalanine mustard. Cancer Res., 51. 2410-24154 1991

Boyer IJ. Toxicity of dibutyltin, tributyltin, and other organotin compounds to human and to experimental animals. Toxicology, 55:253-298. 1989.

Boyer TD and Kenney WC. Preparation, characterization and properties of glutathione-s-transferases, In Zakim D and Vessey D (eds), Biochemical Pharmacology and Toxicology. John Wiley and Sons, New York, NY, pp 297-364 1985.

Boyum A. Separation of leukocyte from blood and bone marrow. Scand.J. Clin. Lab. Invest. 21(S97): 77-89. 1968.

Brown NM. Ph.D. Thesis. Clemson University, USA. 1972.

Brown, P E. Mechanism of action of aminothiol radioprotectors. Nature. 213: 363-364 1967.

Carnetta R, Goodlov J, Smaldone L, Bregman K, Rozencweig M. Pharmacologic Characteristics of carboplatin: clinical experiences. In: Bunn P, Canetta R, Ozols R, Rozencweig M, (eds). Carboplatin: current perspectives and future directions. Philadelphia Saunders: 133-40. 1990.

Cardarelli NF, Cardarelli BM, Libby EP, Dobbins E: Organotin implications in anticarcinogenesis. Effects of several organotins on tumor growth rate in mice Austral J Exp Biol Med Sci 62: 209-214. 1984.

Cardarelli NF, Quitter BM, Allen A, Dobbins E, Libby, EP, Hager P, Sherman LR. Organotin implications in anticarcinogenesis. Background and Thymus involvement. Austral. J Exp. Biol. Med. Sci. 62: 199-208.1984.

Cardin CJ, Roy A. Anticancer activity of organotin compounds. 2. Interaction of diorganotin halides with nucleic bases and nucleosides; the synthesis of adenine, adenosine and 9-methyl-adenine adducts. Inorg Chim Acta 107: 57-61. 1985.

Carrano, AV, Thompson, LH, Lindl PA, and Minkler, J.L. Sister chromatid exchange as an indicator of mutagenesis. Nature 271: 551-553. 1978.

Carrara M, Cima L, Zampiron S, Preti C, Cherki V, Sindellari L In vitro studies on tin-dithio-carbamates Anticancer Res 9 775-780 1989

Caruso F, Bol-Schoenmakers M, Penninks AH Crystal and molecular structure and in vitro antiproliferation and antitumor activity of two Organotin(IV) carbohydrate compounds J of Medicinal. Chem. 36 1168-1174 1993

Chaganti RSK, Schonberg S and German JA Manifold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes Proc Natl acad Sci USA, 71, 4508 1974.

Chakrabarti S, Chakrabarti A, Pal AK Chromosome analysis of Dalton's Lymphoma adapted to swiss mouse Clonal evaluation and C-heterochromatin distribution. Cancer Genetics and Cytogenetics,11. 417-423 1984

Chasseaud LF. The role of glutathione and glutathione-S-transferases in the metabolism of chemical carcinogens and other electroplilic agents Adv Cancer Res. 29, 175-293. 1979

Chatterjee A, Jacob-Raman M, Mohapatra B Potentiation of bleomycin induced chromosome aberrations by the radioprotector-reduced glutathione Mutat Res 214 207-213 1989

Chatterjee, A and Jacob-Raman, M Modifying effect of reduced glutathione on X-ray induced chromosomal aberrations and cell cycle delay in muntjac lymphocytes *in vitro* Mutat Res 175 73-82 1986

Chatterjee A, Chattopadhyay A and Lawlor CJZ. Effect of glutathione on sister chromatid exchanges in normal and buthionine sulfoximine treated mice Mutat Res., 327, 171-177 1995.

Chattopadhyay A, Chatterjee A Modulation of the clastogenic activity of Gamma irradiation in buthionine sulfoximine mediated glutathione depleted mammalian cells Int J of Radiat Biol 75 1283-1291 1999

Chattopadhyay A, Choudhury S and Chatterjee A Modulation of the clastogenic activity of bleomycin by reduced-glutathione, glutathione-ester and buthionine sulfoximine. Mutagenesis 12: 221-225. 1997.

Chow SC, Kass GEN, McCabe MJ, Orrenius . Tributyltin increases cytosolic free Ca^{++} concentration in thymocytes by mobilizing intracellular Ca^{++}, activating a Ca^{++} entry pathway and inhibiting Ca^{++} efflux. Archieves of Biochem. And Biophysics 298: 143-149. 1992.

Ciaccio PJ Tew KD and LaCreta FP. Enzymatic conjugation of chlora with glutathione by human glutathione S-transferases and inhibition by etha acid. Biochem Pharmacol , 42. 1504-1507. 1991.

Clark, E P Thiol-induced biochemical modification of chemo- and radioresponses. Int J Radiat. Oncol Biol. Phys. 12: 1121-1126. 1986

Cleare MJ Coord. Chem Rev., 12, 349. 1974

Cnubben NHP, Rommens AJM, Oudshoorn MJ, van Bladeren PJ Glutathione-dependent biotransformation of the alkylating drug thiotepa and transport of its metabolite monoglutathionylthiotepa in human MCF-7 breast cancer cells. Cancer Res. 58: 4616-4623. 1998.

Codegoni AM Broggini M Pitelli MR, *et al* Expression of genes of potential importance for the response to chemotherapy and DNA repair in patients with ovarian cancer Gyn Oncol 65 130-7 1997

Collyn-D' Hooghe, M, D Hemon, R. Gilet, S. B. Curtis, A. J. Valleron, and E. P Malaise Comparative effects of 60_{Co}-rays and probability of EMT6 cells. A time lapse cinematography study, Int. J. Radiat. Biol., 39,297-306. 1981

Colvin OM, Friedman HS, Gamcsik MP, Fenselau C and Hilton J. Role of glutathione in cellular resistance to alkylating agents. Adv Enzyme Regul. 33: 19-26. 1993

Commandeur JNM, Stijntjes GJ and Vermeulen PE. Enzymes and transport systems involved in the formation and disposition of glutathione-S-conjugates Pharmacol Rev 47 271-330 1995

Cookson MR, Slamon ND, Pentreath VW Glutathione modifies the toxicity of triethyltin and trimethyltin in C6 glioma cells Arch Toxicol 72(4): 197-202, 1998

Crowe AJ, Smith PJ, Cardin CJ, Parge HE, Smith FE: Possible predissociation of diorganotin dihalide complexes - relationship between antitumor activity and structure Cancer Letter 24: 45. 1984

Crowe AJ, Smith PJ, Atassi G Investigations into the antitumor of Diorganotin compounds I Diorganotin Dihalides and Di-pseudohalide complexes Chem Biol Interact 32 171-178. 1980.

Crowe AJ, Smith PJ, Atassi G: Investigations into the antitumor activity of organotin compounds, II Diorganotin dihalide and dipseudohalide complexes Inorg Chim Acta 93: 179. 1984.

Crowe AJ The chemotherapeutic properties of Tin compounds. Drugs Future 12. 255-275 1987

Crowe AJ, Smith PJ, Cardin CJ, Parge HE, and Smith FE. Possible predissociation of diorganotin dihalide complexes-relationship between anti tumor activity and structure. Cancer Lettters: 24-45.1984.

De Santiago A, Aguilar-Santelises M. Organotin compounds decrease in vitro survival, proliferation and differentiation of normal human B lymphocytes. Hum Exp Toxicol: 18: 619-24. 1999.

Dean BJ and Danford N. Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells. In Venitt, S. and Parry, J M.(eds), Mutagenicity Testing. IRL Press, Oxford and Washington DC, pp. 187-232 1984

Deleve LD Kaplowitz N Glutathione metabolism and its role in hepatotoxicity. Pharmacol Ther 52. 287 1991

Dethmers JK and Meister A. Glutathione export by human Lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. Proc. Natl. Acad. Sci. USA. 78: 7492-7496. 1981

Dev-Giri S, Chatterjee A: Modulation of Mitomycin C-induced sister chromatid exchanges and cell cycle delay by buthionine sulfoximine and

reduced glutathione in mouse bone marrow cells in vivo. Mutat Res 413 227-234. 1998

Dirven HAAM, van Ommen B, Bladeren PJ. Involvement of human glutathione s- transferase isozymes in the conjugation of cyclophosphamide metabolites with glutathione Cancer Res 54: 6215-6220. 1994.

Dive C and Hickman JA. Br. J Cancer 64: 192-196. 1991

Doroshow JH, Akman J, Chu F. Role of glutathione- gluathione peroxidae cycle in the cytotoxicity of the anticancer quinones. Pharmacol. Ther. 47, 359-370, 1990.

Eastman A. Cancer Cells, 2: 275-280.1990.

Edgren M, Revesz L Compartmentalized depletion of glutathione in cells treated with buthionine sulfoximine J of Radiology 60: 723-724, 1987

Evans CJ, Karpel S J of Organomet Chem , 16 1 1985

Evans HJ Molecular mechanisms in the induction of chromosome aberrations, In Scott, D , Bridges, B.A., Sobels, F.H. (eds) : "Progress in Genetic Toxicology" Amsterdam . Elsevier/North Holland, p, 57. 1977.

Ferguson LR and Denny WA. Anticancer Drugs: an under estimated risk or an under utilized resource in mutagenesis ? Mutat. Res. 331; 1-26. 1995.

Ferguson LR. Mutagenic properties of anticancer drugs. In Ponder, B.A.J. and Waring M.J. (Eds) The Genetics of Cancer, Kluwer Scientific Publishers, Lancaster, pp177-216. 1995.

Fernandes RS and Cotter TG. Apoptosis or necrosis: intra-cellular levels of glutathione influence mode of cell death. Biochem. Pharmacol. 48:675 1994

Ferrandina G, Scambia G, Damia G, Tagliabue G, Fagotti A, Benedetti Panici P, Mangioni C, Mancuso S, D'Incalci M. Glutathione S-transferase activity in epithelial ovarian cancer: Association with response to chemotherapy and disease outcome. Annals on Oncol. 8: 343-350. 1997.

Fisher DE. Apoptosis in Cancer Therapy : Crossing The Threshold. Cell 78, 539-542. 1994.

Frust A. Chemistry of Chelation in Cancer, Thomas Springfield, New York. P.111. 1963.

Gennari A, Potters M, Seinen W, Pieters RHH: Organotin-induced apoptosis as observed in vitro is nor relevant for induction of thymus atrophy at antiproliferative doses Toxicol and Appl Phar 147: 259-266, 1997.

Ghazal-Aswad S, Hogarth L, Hall AG, *et al.* The relationship between tumor glutathione concentration, glutathione S-transferase isoenzyme expression and response to single agent carboplatin in epithelial ovarian patients. Br. J Cancer 74: 468-73. 1996.

Gielen M, Willem R, Bouhdid A, De Vos D, Kuiper CM, Veerman G, Peters GJ: In vitro antiproliferation effects, toxicity profiles in vivo in mice and antitumor activity in tumor bearing mice of four diorganotin compounds Oncol Rep 3: 583-587, 1996.

Gielen M. Anti tumor active organotin compounds. In: Tin as a Vital Nutrient Implications in Cancer Prophylaxis and other Physiological Processes, Cardarelli, N. F , Ed , CRC Press, Boca Raton, Fla. : 169-173.1986.

Gielen M Tin-based anti tumor drugs. Berlin: Springer-Verlag 1990.

Gielen M, Clercq LD, Willem R, Joosen E. New developments in anti tumor-active of Organotin compounds. In: Tin and Malignant cell Growth. Zuckerman. J. J., Ed. CRC Press Boca Raton Florida. : 39-46.1986.

Gielen M, Willem R, Bouhdid A, De Vos D, Kuiper CM, Veerman G, Peters GJ. In vitro antiproliferative effects, toxicity profiles in vivo in mice and anti-tumor activity in tumor-bearing mice of five organotin compounds. In vivo 9 59-63 1995.

Giese AC Radiations and cell division, Quart Rev Biol 22, 253-282 1947

Gilman A and Philips FS The biological actions and therapetic applications of B-chloroethyl amines and sulfides. Science 103: 409. 1946.

Godwin AK, Meister A, O'Dwyer PJ, Hamilton TC, Huang CS, Anderson ME: High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci USA 89 3070-3074, 1992.

Gonzalez-Beltran, F , Morales-Ramirez, P. In vivo repair during G1 of DNA lesion eliciting sister chromatid exchanges by methylnitrosourea or ethynitrosourea in BrdU subsituted or unsubstituted DNA in murine salivary glands cells. Mutat Res 425: 239-247 1999

Goto K, Akematsu T, Shimagu H, Suigiyama T: Simple differential Giemsa staining of sister chromatids after treatment with photosensitive dyes and exposure to light and the mechanism of staining. *Chromosoma* 53, 223-230, 1975

Goto S, Yoshida K, Morikawa T, Urata Y, Suzuki K and Kondo T. Augmentation of transport for cisplatin-glutathione adduct in cisplatin-resistant cancer cells. Cancer Res, 55: 4297-4301, 1995

Green JA, Vistica DT, Young RC, Hamilton TC, Rogan AM, Ozols RF. Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion Cancer Res 44: 5427-31. 1984.

Griffith OW and Meister A. Translocation of intracellular glutathione to membrane bound γ-glutamyl transpeptidase as a discrete step in the glutamyl cycle: γ-glutathionuria after inhibition of transpeptidase. Proc. Natl Acad. Sci. USA, 76, 268-272. 1979

Gringauz A. Introduction to Medicinal Chemistry, How Drugs act and why. Wiley-VCH 1997

Habig WH., Pabst MJ and Jakoby WB. Glutathione -S- Transferase: The first enzymic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.1974.

Hall AG, Matheson E, Hickson ID, Foster SA, Hogarth L. Purification of an α class glutathione S-transferase from melphalan-resistant Chinese hamster ovary cells and demonstration of its ability to catalyze melphalan-glutathione adduct formation. Cancer Res. 54: 3369-72. 1994.

Hamilton TC, Winber MA, Louie KG, Batist G, Behrens BC, TsuruoT, Grotzinger KR, Mckoy WM, Young RC, Ozols RF. Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and human sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol. 34: 2583-86. 1995.

Hamilton T, O' Dwyer P, Young R, Tew K, Padavic K, Comis R and Ozols R. Phase 1 trial of buthionine sulfoximine (BSO) plus melphalan (L-PAM) in patients with advanced cancer. Proc. A. Meet. Am. Soc. Clin. Oncol., 9: A281, 1990

Havre PA, Yuan J, Hedrick L, Cho KR, Glazer PM. p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res. 55: 4420-4424, 1995.

Hayes JD, McLellan LI Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radical Res 1999.

Hayes JD, Pulford DJ. The glutathione S-tranferase supergene family regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol. 30: 445-600 1995

He SM, Lambert B Induction and persistence of SCE –inducing damage in human lymphocytes exposed to vinyl acetate and acetaldehyde in-vitro. Mutation Research 158: 201-208.1985.

Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metast Rev. 11: 121-129, 1992.

Hollstein M, Sidransky D, Vogelstein B and Harris CC. p53 mutations in human cancers. Science 253: 49-53, 1991.

Holmgren A Glutathione dependent synthesis of deoxyribonucleotides. J Biol. Chem., 258: 3672-3678. (1979).

Howard A and Pelc SR. Synthesis of nucleoprotein in bean root cells. Nature 167: 599, 1951

Hsu TC Cytogenetic Assays of Environmental Mutagens. Allanheld, Osmun & Co Publisher, INC (1982).

Innes JRM. Bioassay of pesticides and industrial chemicals for tumorigenicity in mice. A preliminary note. J. Natl. Cancer Int., 42:1101-1114.1969.

Ishii Y, Bender MA. Effects of inhibitors of DNA synthesis on spontaneous and ultra-violet light-induced sister chromatid exchanges in Chinese hamster cells. Mutat Res 79 19-32. 1982

Ishikawa M, Sasaki KI and Takayanagi Y. Injuries effect of buthionine sulfoximine, an inhibitor of glutathione biosynthesis, on the lethality urotoxicity of cyclophosphamide in mice. J. Pharmacol. Jap., 51: 146-149, 1989.

Ishikawa M, Takayanagi Y and Sasaki KI. modification of cyclophosphomide induced urotoxicity by buthionine sulfoximine and disulfiram in mice Res Commun. Path. Pharmacol , 65: 265-268, 1989.

Ishikawa T The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci 17 463-468. 1992.

Ishikawa T and Ali-Osman F. Glutahione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. J Biol Chem, 268: 20116-20125, 1993.

Ishikawa T, Wright CD, Ishizuka H. GS-X pump is functionally over expressed in cis-diamminedichloroplatinum (II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation. J. Biol. Chem 269: 29085-93 1994.

Jacobson MD, Weil M and Raff MC. Programmed cell death in animal development. Cell, 88 : 347-354, 1997.

Jensson H, Erickson CC, Mannervik B, *et al.* Selective expression of glutathione transferase isozymes in chemically induced preneoplastic rat hepatocytes nodules FEBS Lett 187, 115-120 1985.

Johnson SW, Ozols RF, Hamilton TC. Mechanism of drug resistance in Ovarian Cancer Cancer 71 (suppl): 644-9.1993.

Judson IR. Evaluation of new anticancer drugs. Oncology – A multidisciplinary text book. Horwich A (ed) Chapman and Hall. 1995.

Kano, Y , Fujiwara, Y , Dyskeratosis congenita: survival, sister chromatid exchange and repair following treatments with crosslinking agents. Mutation Res. 103. 327-332. 1982.

Kanzawa F, Hoshi A, Kuretani K, Saneyoshi M and Kawaguchi T. Antitumor activity of 3', 5'- diesters of 5-fluro-2'-deoxyuridine against murine leukemia L1210 cells Cancer Chemother Pharmacol 6 19-23 1981.

Kanzawa F, Nishio K, Kubota N and Saijo N. Antitumor activities of a new indolocarbazole substance, NB-506, and establishment of NB-506- resistant cell lines, SBC-3/NB. Cancer Res 55: 2806-2813. 1995.

Kanzawa F, Sugimoto Y, Minato K, Kasahara K, Bungo M, Nakagawa K, Fujiwara Y, Liu LF and Saijo N. Establishment of a camptothecin analogue (CPT-11)- resistant cell line of human non-small cell lung cancer: characterization and mechanism of resistance. Cancer Res.50: 5919-5924. 1990

Kastan MB, Onyekwere O, Sidransky D, *et al.* Participation of p53 protein in the cellular response to DNA-damage. Cancer Res 51:6304-6311.1991.

Kato H and Shimada H. Sister chromatid exchanges induced by mitomycin C: A new method of detecting DNA damage at chromosomal level. Mutat Res 28 459-464, 1975

Keppler BK, Ed Metal Complexes in Cancer Chemotheraphy. New York V.C.H Weinheim 261-96 1993

Kerr JF, Wyllie AH, Currie AR Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer, 26:239-257, 1972.

Kilmman BA Caffeine and Chromosomes. Elsevier, Amstredam. 1977

Koch H, Smith ER, Shimp NF, Connor J: Analysis of trace elements in human tissue· 1 Normal Tissue. Cancer 9: 399-411, 1956.

Kopf H and Kopf-Maier P Tumor inhibition by metallocene dihalides of early transition metals chemicals and biological aspects, in Platinum, Gold and other Metals Chemotherapeutic Agents: Chemistry and Biochemistry, Lippard SJ. (ed). ACS Symp. Ser. No. 209, American Chemical Society. Washington, D.C , 315 1983.

Kosower NS and Kosower EM. The glutathione status of cells. Int. Rev. Cytol , 54, 109, 1978

Kratz F, Schutte MT Anticancer metal complexes and tumor targeting strategies. The Cancer Journal 11: 60-67, 1998.

Kubbutat MHG and Vousden KH Keeping an old friend under control: regulation of p53 stability Mol Med Today 4: 250-256. 1998.

Kuerbitz SJ, Plunkett BS, Walsh WV. *et al.* Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Sci. USA. 89:7491-7495.1992

Lai G, Ozols R, Young R and Hamilton T. Effect of glutathione on DNA-repair in cisplatin resistant human ovarian cancer cell lines J. Natl. Cancer Inst. 81: 535. 1989.

Larsson A, Orrenius S, Holmgren A, Mannervik B(eds). Functions of Glutathione; Biochemical, Physiological, Toxicological and Clinical aspects New York. Raven Press 1983

Latt SA Sister chromatid exchanges, indices of human chromosome damage and repair detection by fluorescence and indiction by mitomycin C Proc Natl Acad Sci USA 71 3162-3166, 1974

Lee FYF Alluris-Turner MJ, Siemann DW Depletion of tumor versus normal tissues of glutathione by buthionine sulfoximine. British Journal of Cancer 56 33-38. 1987

Levine AJ, Momand J and Finlay CA. The p53 tumor supressor gene. Nature, 351: 453-456, 1991.

Levine AJ. p53 , the cellular gatekeeper for growth and division. Cell 88: 323-331 1997

Lewis AD, Hayes JD, Wolf CR. Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: Intrinsic differences and cell cycle effects. Carcinogenesis 9:1283-7. 1988.

Linn SC, Honkoop AH, Hoekman K, Valk PVD, Pinedo HM, Giaccone G. p53 and P-glycoprotein are ofter co-expressed and are associated with poor prognosis in breast cancer Brit J Cancer. 74:63-68.1996

Lissuer II Zwei Falle von Leukaemie. Berl Klin 40: 403. 1865.

Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53 Cell 70 923-935, 1992

Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature, 362: 847-849, 1993

Lowry OH Rosenbrough NJ, Farr L and Randall RJ. Protein measurement with Folin phenol reagent J Biol Chem 193 265-275, 1951

Lu X Lane DP Differential induction of transcriptionally active p53 following UV or ionizing radiation Defects in chromosome instability syndromes? Cell 75 765-778,1993

Lutzky J, Astor MB Taub RN, Baker MA, Bhalla K, Gervasoni JE, Rosoado M, Stewart V, Krishna S, Hindenberg AA· Role of glutathione and dependent enzymes in anthacycline-resistant HL60/AR cells. Cancer Res 49:4120-4125, 1989

MacRae, W D , MacKinnon, E A , Stich, H.F. he fate of U.V -induced lesions affecting SCEs, chromosome aberrations and survival of CHO cells arrested deprivation of arginine Chromosoma 721: 15-22. 1979.

Makovie S, Voehringer DW, Story MD, McConkey DJ, McDonnell, Meyn RE. Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is increased by depleting cellular thiols. Oncogene 15: 1461-1470. 1997.

Mannervik B. The isoenzymes of glutathione-s-transferases. In Meister, A.(eds), Advances in Enzymology John Wiley and Sons, New York, NY, pp 357-417 1985

Martensson JM Jain A Stole E, Frayer W, Auld PAM and Meister A Inhibition of glutathione synthesis in newborn rat, a model of endogenously produce oxidative stress Proc Natl Acad Sci USA 88 9360-9364, 1991

Mattern J and Volm M Multiple pathway drug resistance (review). Int. J 2 557-561, 1993

Mazia D. Mitosis and the physiology of cell division.In the Cell. (Eds) Brachet, J. and Mirsky A.R. 3, 251, Academic Press New York.1961.

McGuire WP Treatment of epithelial Ovarian malignancies. Cancer 71 (suppl) 1541-50 1993

McLellan LI Glutathione and glutathione-dependent enzymes in cancer drug resistance Drugs Resistance Updates 2, 153-164 1999

Meijer C, Mulder NH Timmer-Bosscha H, Sluiter WJ, Meersma GJ, de Vries EG Relationship of cellular glutathione to the cytotoxicity and resistance to seven platinum compounds. Cancer Res. 52: 6885-6889, 1992.

Meister A Glutathione deficiency produced by inhibition of its synthesis and its reversal applications in research and therapy. Pharmacol. Ther. 51: 155-194.1991.

Meister A. Glutathione metabolism and its selective modification. J. Biol. Chem 26, 17205-17208, 1988

Meister A Glutathione, Ascorbate, and Cellular Protection. Cancer Research (suppl) 54 1969s-1975s 1994

Meister A. Novel drugs that affect glutathione metabolism. In: Woolley,P V and Tew,K.D. Eds Mechanism of Drug Resistance in Neoplastic Cells. Bristol Myers Symposium No. 9, Chapter 7, pp. 99-127, New York Academic Press 1988

Meister A and Griffith OW Effects of methionine sulfoximine analogs on the synthesis of glutamine and glutathione; possible chemotherapeutic implications Cancer Treat Rep. 63: 1115-1121. 1979.

Meister A Glutathione metabolism and its selective modification. J. Biol. Chem , 263. 17205-17208, 1988.

Meister A, Glutathione metabolism and transport. In: Nygaard, O.F., and Simic, M.G. Eds. Radioprotectors and Anti-Carcinogens. pp 121-151. New York: Acadamic Press 1983.

Meyer DJ, Gilmore KS, Harris JM, Hartley JA and Ketterer B. Chbucil-monoglutathione conjugate is sequestered by human α class glutathione S-trasferases. Br J Cancer, 66 433-438, 1992.

Meyn RE, Stephens LC, Mason KA and Medina D Radiation-induced apoptosis in normal and pre-neoplastic mammary glands *in vivo* significance of gland differentiation and p53 status Int J. of Cancer, 65 466-472, 1996

Miller EC and Miller JA. Mechanisms of Chemical Carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. Pharmacol. Rev. 18 805-838 1966.

Milner J The p53 tumour suppressor. In Signal Transduction Helden CH, Purton M (eds) Chapman and Hall 1996

Mistry P, Kelland LR, Abel G *et al.* The relationships between glutathione, glutathione S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines Br J Cancer 64 215-20 1991

Mitelman F. Catalog of chromosome aberrations in cancer. 5th ed Wiley-Liss, New York.

Moebus VJ, Stein R, Kieback DJ, Runnebaum IB, Sass G, Kreienberg R. Anti tumor activity of a new organomettallic compounds in human ovarian cancer cell lines and comparison to platin derivatives. Anti Cancer Res. 17:815-22.1997.

Moscow JA, Townsend AJ, Cowan KH. Elevation of π class glutathione-S-transferase activity in human breast cancer by transfection of the GST-π gene and its effect on sensitivity to toxins. Mol. Pharmacol 36: 22-28, 1989.

Mulay I, Roy R, Knox BE, Suhr NH, Delaney WE: Trace metal analysis of cancerous and non-cancerous human tissue. J Natl Cancer Inst 47:1-13, 1971

Murray GI, Burke MD, Ewen SW: Glutathione localization in benign and malignant human breast lesions. Br J Cancer 55: 605-609, 1987.

Musmeci MT, Madonia G, Lo Giudice, Silvestri A, Ruisi R, Barbieri R. Interaction of organotins with biological systems Appl. Organomettallic. Chem 6: 127-138, 1992.

Nagasawa H, Robertson CS, Arundel and Little JB The effect of X-irradiation on the progression of mouse 10 T 1/2 cells, released from density-inhibited cultures, Radiation Research, 97, 537-545, 1984

Nakanishi Y, Matsuki A, Takayama K, Yatsunami J, Kawasaki M, Abe M, Hara N. Glutathione derivatives enhance Adriamycin cytotoxicity in human lung adenocarcinoma cell line. Anticancer Res. 17: 2129-2134. 1997

Narayanan VL, in Reinhoudt DN, Connors TA, Pinedo HM and van de Poll KW. (Eds). Srructure-activity relationships of antitumor agents, Martinus Nijhoff, The Hague, p 5. 1983.

Nasmyth K. Viewing· putting the cell cycle in order. Science 274: 1643-1645, 1996

Niitsu Y, Takahashi Y, Saito T Serum glutathione S- transferase II as a tumor marker for gastrointestinal malignancies. Cancer 63, 317-332. 1989

Nishi, Y , Hasegawa, M M., Taketomi, M., Ohkawa, Y., Inui, N. Comparison of 6-thioguinine-resistant mutation and sister chromtid exchanges in Chinese hamster V79 cells with forty chemical and physical agents. Cancer Res., 44: 3270-3279.1984

Ohmori T, Morikage T, Sugimoto Y, Fujiwara Y, Kasahara K, Nishio K, Ohta S, Sasaki Y, Takahashi T and Saijo N. The mechanism of the difference in cellular uptake of platinum derivatives in non-small cell lung cancer cell line(PC-14) and its cisplatin-resistant subline(PC-14/CDDP). Jpn J Cancer Res 84: 83-92. 1993.

Ono K and Shrieve DC. Enhancement of EMT6/SF tumor cell killing by mitomycin C and cyclophosphamide following *in vivo* administration of buthionine sulfoximine Int J Radiat Oncol Biol Phys 12· 1175-78 1986

Oren M Regulation of the p53 tumor suppressor protein J. Biol Chem 274 36031-36034 1999

Otera J, Loka S and Nozaki H J Org. Chem.54: 4013. 1989.

Ozols RF, Hamilton TC, Masuda H and Young RC. Manipulation of cellular thiols to influence drug resistance In Wolley, P.V. and Tew, K D Eds Mechanisms of drug Resistance in Neoplastic cells. Bristol Myers Symposium No 9, Chapter 19, pp. 289-305. New York: Academic Press.1988.

Painter RB. A replication model for sister-chromatid exchange, Mutation Res., 337-341. 1980

Peiters RHH, Bol M, Seinen W, Penninks AH Cellular and molecular aspects of organotin-induced thymus atrophy Human and Experimental Toxicology 13 876-879 1994

Pendyala L Perez R Weinstein A, Zdanowicz J and Creaven PJ Effect of glutathione depletion on the cytotoxicity of cisplatin and iproplatin in human malanoma cell lines Cancer Chemother Pharmacol. 40: 38. 1997.

Penninks AH, Bol-Schoenmakers M, Seinen W. Cellular interactions of organotin compounds in relation to their anti-tumor activity. In Tin-based anti-tumor drugs: Gielen M (ed). Nato ASI series, Series H: Cell Biolgy; Springer-Verlag: Berlin. 37:169-170. 1990.

Perez RP, Hamilton TC and Ozols RF. Resistance to alkylating agents and cisplatin insights from ovarian carcinoma model systems. Pharmacol Ther 48, 19-27 1990

Perez RP, O'Dwyer PJ, Handel LM, Ozols RF, Hamilton TC Comparative cytotoxicity of CI-973, cisplatin, carboplatin and tetraplatin in human ovarian carcinoma cell lines Int J Cancer , 48 265-9 1991

Perry P and Evans HJ Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange Nature 258 121-125, 1975

Perry, P , Wolff, S , New Giemsa method for the differential staining of sister chramatids Nature 251 156-158, 1974

Perego P, Righetti SC, Supino R, Delia D, Caserini C, Carenini N, *et al* Role of apoptosis-related proteins in the cisplatin-resistant phenotype of human tumor cell lines Apoptosis 2 540-548 1997

Peters WHM and Roelofs HMJ Biochemical characterization of resist mitoxantrone and Adriamycin in Caco-2 human colon adenocarcinoma a possible role of glutathione S- transferases Cancer Res , 52 1886-1890, 1994

Povirk LF and Austin MJF Genotoxicity of bleomycin Mutat Res 257 127-143 1991

Prestayko AW Cisplatin a Preclinic view In Cisplatin Current Status andnew Developments, Prestayko, A W , Crooke, S T , Carter, S K , Eds , Acadrmic Press New York, 1-7,1980

Prochaska HJ and Talalay P Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver Cancer Res , 48 4776-4782, 1998

Prochaska HJ, De Long MJ and Talalay P. On the mechanism of induction of cancer protective enzymes a unifying proposal. Proc. Natl. Acad. Sci USA, 82 8232-8236,1985

Raikar A and Ali-Osman F. Modulation of DNA ligase activities in BCNV and cisplatin resistant human malignant astrocytoma cell-line by glutathione depletion. Proc. Am Assoc. Cancer Res. 33: 7. 1992.

Rainaldi R and Mariani T. The distribution of induced sister chromatid exchanges: a tool for identifying agents directly interacting with DNA. Mutat. Res. 103: 333-337. (1982).

Raposa T. SCE and chemotherapy of non-cancerous and cancerous condition, In: Sandberg A.A. (ed). "sister chromatid exchange" New York, Alan R. Liss, Inc. pp.579-618. 1982.

Raunio H, Husgaluel-Pursiainen K, Anttila S. Diagnosis of polymorphism in carcinogen-activating and inactivating enzymes and cancer susceptibility. Gene,1, 113-120, 1995.

Ray JH and German J. Sister chromatid exchanges in the chromosome breakage syndrome ,In: Sandberg A.A. (ed). "sister chromatid exchange" New York, Alan R. Liss, Inc. pp.553-573. 1982.

Reedijk J, Fichtinger Schepman AJ, van Oosterom A, *et al.* Platinum ammine coordination compounds as antitumuor drugs. Molecular aspects of the mechanism of action. Structure and Bonding 67, 54-89, 1987.

Respondek J, Engel J. Organometallics in medecine. Drugs of the future; 21, 391-408. 1996.

Rice CD, Banes MM, Ardelt TC. Immunotoxicology in channel catfish, Ictalurus punctatus, following acute exposure to tributyltin. Archives of Environmental Contamination and Toxicology 28: 464-470. 1995.

Robichand NJ and Fram RJ. Schedule dependance of buthionine sulfoximine in reversing resistance to cisplatin. Chem. Biol. Interact 76: 333.1990.

Richon VM, Schulte N and Eastman A. Multiple mechanisms of resistance to cis-diamminedichloroplatinum (II) in murine leukemia L1210 cells. Cancer Res 47: 2056-2061. 1987.

Rosenberg B, VanCamp L, Trosko JE and Mans VH. Platinum compounds: A new class of potent antitumor agents. Nature 222: 385-386. 1969.

Saburi Y, Nakagawa M, Ono M *et al.* Increased expression of glutathione S-transferase gene in cis-diamminedichloroplatinum (II)-resistant variant of a Chinese Hamster ovarian cell line. Cancer Res. 49: 7020-5. 1989.

Saher, E., Kittrel, C., Fulghum,S., Feld, M., Latt, S.A., Sister chromatid exchange induction in Chinese hamster ovary cells by 8-methoxypsoralen and brief pulses of laser light. Mutat. Res. 1983. 91-105. 1981.

Sasaki MS. Sister chromatid exchanges as a reflection of cellular DNA repair. In Sandberg, A.A.(Ed) Sister chromatid exchange. Alan L. Liss, New York, NY pp, 35-164. (1982).

Sasaki YF, Yamaha H, Sugiyama C and Kinae N. Increasing effect of tri-n-butyltins and triphenyltins on the frequency of chemically induced chromosome aberrations in cultured Chinese hamster cells. Mutation Research, 300: 5-14. 1993.

Saxena A K. Appl. Organomet. Chem., 1: 39 1987.

Schabel FM Jr., Griswold DP Jr., Corbett TH, Laster WR Jr., Mayo JG and Lloyd HH. Testing Therapeutic Hypothesis in mice and man: Observation of the therapeutic activity against advanced solid tumors of mice treated with anticancer drugs that have demonstrated of potential clinical utility for treatment of advanced solid tumors in man. Methods in Cancer Research, Vol. XVII. Academic Press, Inc. 1978.

Sieber SM and Adamson RH. Toxicity of antineoplastic agents in man: Chromosomal aberration, antifertility effects and carcinogenic potential. Adv. Cancer Res. 22· 57-155. 1975.

Shafer DA Alternate replication bypass mechanisms in sister chromatid exchange formation In Sandberg A.A (ed). "sister chromatid exchange" New York, Alan R Liss, Inc pp 67-98 1982.

Sharma M, Tomasz M. Conjugatione of glutathione and other thiols with bioreductively activated mitomycin C :effect of thiols on the reductive activation rate. Chem. Res. Toxicol. 7 1994.

Shaw JP and Chou INJ. Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. J. Cell Physol.: 129, 193-198. 1986

Sherman LR and Huber F. Relationship of cytotoxic groups in organotin molecules and the effectiveness of the compounds against leukemia. Applied Organomet. Chem. 2;65-72. 1987.

Sherman SE, Lippard SJ Structural aspects of platinum anticancer drug interactions with DNA Chem. Rev 87, 1153-1181. 1987

Shiraishi Y and Sandberg AA. Effects of various chemical agents on sister chromatid exchanges, chromosomal aberrations, and DNA repair in normal and abnormal human lymphoid cell lines. J. Natl. Cancer Inst. 62: 27-35, 1979.

Shrieve DC, Denekamp J. Minchinton AI: Effects of glutathione depletion by buthionine sulfoximine on radiosensitization by oxygen and misonidazole in vitro. Rad Res 102: 283-294, 1985.

Siebenlist KR and Taketa F. Toxicol. Appl. Pharmacol. 58: 67. 1981.

Sies, H. Oxidative stress: from basic research to clinical application. Am. J. Med., 91: 31S-38S, 1991.

Singh PP and Sharma KK. Topological aspects of the biological activity of organotin compounds. Indian J. Chem. 32B; 551-554.1993.

Snedecor, G.W., Cochran W.G. Statistical methods, 6^{th} Edn. Iowa state Press, Ames IA. 1967.

Speit, G., Wolf, M. Vogel, W. The effect of sulphydryl compounds on sister chromatid exchanges. Mutat.Res. 78:267-272. 1980.

Stephenou, G., Vlastos, D. Vlachodimitropoulos, D., Demopoulos N.A. A comparative study on the effect of MNU on human lymphocyte cultures in vitro evaluated by O^6-mdG formation, micronuclei and sister chromatid exchanges induction. Cancer Letters 109: 109-114, 1996.

Stetka DG and Wolff S. Sister chromatid exchanges as an assay for genetic damage induced by mutagens-carcinogens. I&II. Mutat. Res. 41: 333-350, 1976.

Stridh H, Orrenius S, Hampton MB. Caspase involvement in the induction of apoptosis by the environmental toxicants tributyltin and triphenyltin. Toxicol Appl Pharmacol 156: 141-146, 1999.

Suthanthiran M, Anderson ME, Sharma VK and Meister A. glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via CD2 and CD3 antigens. Proc. Natl. Acad. Sci. USA. 87: 3343-3347, 1990.

Suzukake K, Petro BJ and Vistica DT. Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. Biochem. Pharmacol., 31, 121-124, 1982.

Suzukake K, Vistica BP and Vistica DT. Dechlorination of L-phenylalanine mustard by sensitive resistant tumor cells and its relationship to intracellular glutathione content. Biochem. Pharmacol., 32: 165-167, 1983.

Symonds H, Krall L, Remington L, *et al.* p53 dependent apoptosis suppress tumor growth and progression *in vivo.* Cell 78: 703-711, 1994.

Talalay P, DeLong MJ and Prochaska HJ. Molecular mechanisms in protection against carcinogenesis. In: J. G. Cory and A. Szentivani(eds.), Cancer Biology and Therapeutics, pp. 197-216. New York: Plenum Publishing Corp., 1987.

Taniguchi N, Higashi T, Sakamoto Y, Meister A. Glutathione Centennial: Molecular prospectives and Clinical Implications. Academic Press, California. 423-433, 1989.

Thompson TA, *et al.* Induction of apoptosis by organotin compounds in vitro Neuronal protection with antisense oligonucleotides directed against stannin. Journal of Pharmacology and Exp. Therapeutics 276: 1201-1215, 1996.

Thompson TC, Park SH, Timme TL, *et al.* Loss of p53 function leads to metastasis in ras + myc- initiated mouse prostrate cancer. Oncogene, 10 : 869-879, 1995.

Tsuchida S and Sato K. Glutathione transferases and cancer. Crit. Rev. Biochem. Mol. Biol., 27, 337-384.1992.

Tucker JD, Auletta A, Cimino MC, Dearfield KL, Jacobson-Kram D, Tice RR and Carrano AV. Sister-chromatid exchang: Second report of the Gene-Tox program, Mutation Res., 297, 101-180,1993.

Van der Zee AGJ, van Ommen B, Meijer C, Hollema H, van Bladeren PJ, de Vries EGE. glutathione S-transferase activity and isoenzyme position in benign ovarian tumors, untreated malignant ovarian tumor, malignant ovarian tumors after platinum/cyclophosphamide chemotherapy. J. Cancer, 66:930-936, 1992.

Vogelstein B, Kinzler KW. p53 function and dysfunction. Cell. 70:523-526,1992.

Vral A, Cornelissen M, Thierens H, Louagie H, Philippe J, Strijckmans K, Ridder DL: Apoptosis induced by fast neutrons versus ^{60}Coγ-rays in human peripheral blood lymphocytes. Int J Radiat Biol 73: 289-295, 1998.

Wang XW. Role of p53 and Apoptosis in Carcinogenesis. Anticancer research 19: 4759-4771, 1999.

Wang XW, Gibson MK, Vermeulen W, *et al.* Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. Cancer Res. 55: 6012-6016, 1995.

Wang XW, Yeh H, Schaeffer L, *et al.* p53 modulation of TFIIH-associated nucleotide excision repair activity. Nature Genet. 10: 188-195, 1995.

Wardman, P., Dennis, M.F., Stratford, M.R.L., White, J. Extracellular: Intracellular and subcellular concentration gradients of thiols. Int.J. Radiat. Oncol. Biol. Phys. 22: 751-754. 1992.

Waxman DJ. Glutathione S-transferase: role in alkylating agent resistance and target for modulation chemotherapy-a review. Cancer Res, 50: 6449-6454, 1992.

Weinberg RA. Oncogenes and Molecular origins of Cancer. Cold Spring Harbour Laboratory Press, Cold Spring Harbour. NY. 1989.

Weinberg RA. How cancer arises. Sci. Am., 275: 62-70. 1996.

Weinstein JN, Myers TG, O'Conner PM, Friend SH, Fornace AJ Jr., Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, vanOsdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE and Paull KD. An information-intensive approach to the molecular pharmacology of cancer. Science, 275: 343-349, 1997.

Wrigley EC, McGown AT, Buckley H, et al. Glutathione S-transferase activity and isoenzyme levels measured by two methods in ovarian cancer, their value as markers of disease outcome. Br. J. Cancer 73: 763-9. 1996.

Wyllie AH. Glucocorticoid-induced thymocyte apotosis is associated with endogenous endonuclease activation. Nature, 284 : 555-556, 1980.

Yonish-Rouach E, Resnitsky D, Lotem J, Sachs L, Kimichi A, Oren M. Wild-type p53 induced apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352: 345-347, 1991.

Zheng, S., Newton, G.L., Gonick, G, Fahey, R.C., Ward, J.F. Radioprotection of DNA by thiols: relationship between the net charge on a thiol and its ability to protect DNA. Radiat. Res. 114: 11-27. 1988.

List of Publication:

- Basu Baul S, Basu Baul TS, Rivarola E, Daktemieks D, Tiekink ERT, Syna-ai C, Chatterjee A. Synthesis and characterization of diorganotin(IV) complexes of N-(2-Pyridylmethylene)-arylamines and Mutagenicity testing *in vivo* of Et_2SnCl_2. [L^4 = N-(2-Pyridylmethylene)-4-toluidine]. **Appl Organometallic Chem. 12: 503-513, 1998.**

- Syng-ai C, Basu Baul TS, Chatterjee A. Antiproliferative and cytotoxic effect of a novel organotin compound on mammalian cells both *in vivo* and *in vitro*. **Mutation Research, 2001. In press.**

- Syng-ai C, Basu Baul TS, Chatterjee A. Inhibition in cell proliferation and preliminary studies on anti-tumor activity of a novel organotin compound. **J. Environ. Pathol. Toxicol. and Oncol. Vol. 20, Nov 2001. In press.**

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Synthesis and Characterization of Diorganotin(IV) Complexes of *N*-(2-Pyridylmethylene)-arylamines and Mutagenicity Testing *in vivo* of $Et_2SnCl_2 \cdot [L^4 = N\text{-}(2\text{-Pyridylmethylene})\text{-}4\text{-toluidine}]$

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Diorganotin(IV) dichloride complexes of the type $R_2SnCl_2 \cdot L$ (R = methyl, ethyl, vinyl, t-butyl, n-butyl or phenyl; L = *N*-(2-pyridylmethylene)arylamine) have been synthesized and characterized on the basis of IR, NMR and ^{119}Sn Mössbauer studies. Investigation of the complexes indicated that *N*-(2-pyridylmethylene)arylamines form distorted *trans*-octahedral complexes with R_2SnCl_2 similar to the well-known $R_2SnCl_2 \cdot L$. Cytogenetic toxicology testing has been performed for $Et_2SnCl_2 \cdot L^4$ [L^4 = *N*-(2-pyridylmethylene)-4-toluidine] in mouse bone-marrow cells *in vivo* since such testing is a regulatory requirement before new drugs are released. This tin compound induced delay in cell-cycle kinetics and sister chromatid exchanges (SCEs) significantly. The effect of $Et_2SnCl_2 \cdot L^4$ was greater when endogenous glutathione (GSH) was depleted by buthionine sulphoximine (BSO). It seems that $Et_2SnCl_2 \cdot L^4$ induces SCEs due to formation of adduct by binding on DNA which could interfere in DNA synthesis and cause delay in cell proliferation. Depletion of GSH could reduce the shielding effect of GSH on chromatin and allows more $Et_2SnCl_2 \cdot L^4$ to bind on DNA. © 1998 John Wiley & Sons, Ltd.

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INTRODUCTION

Diorganotin(IV) compounds, R_2SnCl_2 (X = anion) are often tetrahedral, and when appropriate nitrogen-chelating ligands are co-ordinated to the central metal, octahedral complexes $R_2SnCl_2 \cdot L$ (L = bidentate ligand) are obtained.[1,2] These complexes structurally resemble the active platinum compounds, i.e. *cis*-diamminedichloroplatinum(II) (cisplatin) and *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II) (carboplatin), and consequently a large number of such complexes have been tested for antitumour and anticancer activity. A structural correlation with biological activity for diorganotin complexes has shown that active

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