Chapter 4.

Does $\text{Et}_2\text{SnCl}_2 \cdot L \ {L=N-[p-(2-}$

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 0.81 g (NaCl; Merck India Ltd.)
(c) Dipotassium Hydrogen Phosphate 0.06g (K₂HPO₄; Merck India Ltd.)
(d) Methyl - p - 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: Hycione, USA.)

Methods:

DL cells viability was measured after 7 days of intraperitoneal transplantation by counting cells stained in Trypan blue after 2, 4, 17, and 24 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, Ca²⁺ and Mg²⁺- 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-lmara 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-lmara 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.
Markovic 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
demonstrate 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.

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

\(^*\) \text{p}<0.001 2\times2 contingency \(\chi^2\) - Test; compared with control value.

\(\dagger\) \text{p}<0.05 2\times2 contingency \(\chi^2\) - Test; compared with OTC (24h) only.
Table 4.2. Percentage of Apoptotic cells observed after treatment with OTC (15mg kg\textsuperscript{1}) alone or in combination with BSO (50mg kg\textsuperscript{-1}) in Dalton's Lymphoma Cells.

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

In each sample at least 1000 cells were considered.

* \( p<0.001 \) 2x2 contingency \( \chi^2 \) - Test; compared with their control value.

\( ^* p<0.01 \) 2x2 contingency \( \chi^2 \) - 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.
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 dithio 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 Å which in turn would determine the formation of tin-DNA complex. The present new compound \(\text{Et}_2\text{SnCl}_2 \cdot \text{L} \quad (L=N-[p-(2-pyridyl)methylene] methylbenzenamine}) \) (OTC) has an Sn-N bond length of 2.46 Å which is larger than 2.39 Å 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 intraperitoneally 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 intraperitonal transplantation in 3 months old Swiss albino mice using an inoculums size of 10⁶ 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 CA 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