Chapter 3.

Anti Tumor Activity of Et₂SnCl₂L {L=N-[p-(2-pyridyl)methylene]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 R₂SnX₂·2L (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-carcinogenicity. 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 Ph₃SnCl. 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 2.39Å show anti-tumor activity whereas those smaller than 2.39Å 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 anticancer 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$_2$SnCl$_2$.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$_2$SnCl$_2$.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:** $1 \times 10^6$ Dalton's Lymphoma cells were inoculated and the survival time of each animal was recorded.

**Group 2:** $1 \times 10^6$ Dalton's Lymphoma cells were inoculated followed by OTC treatment on Day 1, 5, and day 9 at a dose of $15 \text{ mg kg}^{-1}$. 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 $50 \text{ mg kg}^{-1}$ 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 1st, 5th, and 9th day after transplantation of tumor cells. On the 10th 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 (0.01 μg 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 1ˢᵗ, 5ᵗʰ and 9ᵗʰ 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 4ᵗʰ and 8ᵗʰ 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-pyridyl)methylene] 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.

<table>
<thead>
<tr>
<th>Exp. Condition</th>
<th>No. of animals</th>
<th>BSO treatment (days)</th>
<th>OTC treatment (days)</th>
<th>Median survival time(days)</th>
<th>Range survival time(days)</th>
<th>T/C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>10-16</td>
<td>-</td>
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<tr>
<td>BSO</td>
<td>02</td>
<td>4(^{th}), 8(^{th})</td>
<td>-</td>
<td>13</td>
<td>11-13</td>
<td>100</td>
</tr>
<tr>
<td>OTC</td>
<td>11</td>
<td>-</td>
<td>1(^{st}), 5(^{th}), 9(^{th})</td>
<td>19</td>
<td>14-28</td>
<td>146</td>
</tr>
<tr>
<td>BSO+OTC</td>
<td>10</td>
<td>4(^{th}), 8(^{th})</td>
<td>1(^{st}), 5(^{th}), 9(^{th})</td>
<td>25</td>
<td>19-31</td>
<td>192</td>
</tr>
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</table>

Cisplatin 1mg kg\(^{-1}\)

<table>
<thead>
<tr>
<th>Exp. Condition</th>
<th>No. of animals</th>
<th>BSO treatment (days)</th>
<th>OTC treatment (days)</th>
<th>Median survival time(days)</th>
<th>Range survival time(days)</th>
<th>T/C %</th>
</tr>
</thead>
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<tr>
<td></td>
<td>05</td>
<td>-</td>
<td>1(^{st}), 5(^{th}), 9(^{th})</td>
<td>35</td>
<td>22-39</td>
<td>269</td>
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</table>

* 24 hours before OTC treatment.

Table 3.2 Effect of cisplatin and OTC on chromosomal aberration induction in mouse bone marrow cells

<table>
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<tr>
<th>Cisplatin (1mg kg(^{-1}))</th>
<th>OTC (15mg kg(^{-1}))</th>
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<tr>
<td>TM Ab. M(%)</td>
<td>TM Ab. M(%)</td>
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<tr>
<td>116 1.75</td>
<td>128 1.56</td>
</tr>
<tr>
<td>106 2.80</td>
<td>103 2.91</td>
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<td></td>
<td>201 3.98</td>
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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.

<table>
<thead>
<tr>
<th>Expt. Condition</th>
<th>TM</th>
<th>M1%</th>
<th>M2%</th>
<th>Mean M1% ± SEM</th>
<th>AGT (H)</th>
<th>Mean AGT ± SEM</th>
</tr>
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<tr>
<td>Untreated</td>
<td>099</td>
<td>079</td>
<td>21</td>
<td>40</td>
<td>37</td>
<td>39.25 ± 0.75</td>
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<td></td>
<td>085</td>
<td>082</td>
<td>18</td>
<td>40</td>
<td>40</td>
<td>46.50 ± 0.95</td>
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<td></td>
<td>090</td>
<td>076</td>
<td>24</td>
<td>40</td>
<td>40</td>
<td>46.50 ± 0.95</td>
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<td></td>
<td>106</td>
<td>071</td>
<td>29</td>
<td>77 ± 2.34</td>
<td>37</td>
<td>39.25 ± 0.75</td>
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<tr>
<td>OTC</td>
<td>078</td>
<td>086</td>
<td>14</td>
<td>44</td>
<td>44</td>
<td>44.50 ± 0.95</td>
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<td>079</td>
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<td>06</td>
<td>46</td>
<td>46</td>
<td>46.50 ± 0.95</td>
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<tr>
<td></td>
<td>091</td>
<td>100</td>
<td>00</td>
<td>48</td>
<td>48</td>
<td>46.50 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>075</td>
<td>100</td>
<td>00</td>
<td>95 ± 3.31*</td>
<td>48</td>
<td>46.50 ± 0.95</td>
</tr>
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</table>

TM; Total Metaphases. M1; 1st cycle metaphases.

* p<0.05 2x2 contingency χ² – 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.
Fig. 3.2

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