CHAPTER - 5
Fate of levamisole in cell culture
And
$^3$H-levamisole binding to murine splenic lymphocytes, normal and malignant human lymphocytes.
In all the experiments described thus far myeloma cells were incubated with varying concentrations of levamisole. Although effect of levamisole has been observed in a dose dependent manner the effective concentration of levamisole to bring about the observed effect is not known. Levamisole, being a freely water soluble compound, it is expected that its entry into the cells through the lipid bilayer will be meagre. Also as fetal calf serum is used for cell culture it is possible that levamisole can bind to the serum proteins. Hence in this chapter it is attempted to determine the fate of levamisole in cell culture by estimating the concentration using HPLC at different culture time periods.

Further the interaction of levamisole with myeloma cells was studied by estimating the binding of $^3$H-levamisole to whole cells and broken cell preparations.

Levamisole has been shown to bind avidly to proteins. The binding of levamisole to total plasma proteins of 6 animal species was determined in vitro by equilibrium dialysis. The amount of drug bound to plasma proteins was independent of levamisole concentration. Levamisole was bound to a limited extent to plasma proteins of animal species studied (Sahagun et al 1997). Levamisole has been shown to be decomposed enzymatically / non-enzymatically into three degradation products (Kimberly et al 1991). The structures of the purified products are A) 3-(2-mercaptoethyl)-5-phenyl-imidazolidine-2-one (OMPI) B) 6-phenyl-2,3-dihydroimidazo (2,1-b) thiazole and C) bis(3-(2-oxo-5 phenyl imidazolidine-1-yl) ethyl] disulfide. The decomposition of levamisole has been shown to be temperature and pH dependent. Hence, the free and unaltered levamisole concentration available for interaction with cells could be very low as compared to the final concentration in cell culture as a result of protein binding and degradation. Apart from decomposition, other reason for the high concentration of levamisole needed in cell culture to get the effects could be due to low binding of levamisole to the cells. Some of the metabolites of levamisole which have been characterized are given below.
**Objectives:**

1. To determine the stability of levamisole in culture with and without FCS.
2. To determine the amount of levamisole bound to normal B cells, mitogen-activated B cells and myeloma cells.

**Methods:**

1. **Analysis of Levamisole by HPLC**

   **A. Cell culture and extraction of levamisole**

   Myeloma cells, $0.25 \times 10^6$/ml were cultured with and without FCS and levamisole was added at a concentration of 2.5 mM. At 0, 24, 48, 72h of culture...
period, 1 ml cell suspension was taken and centrifuged at 500 g and the supernatant was collected. Levamisole was extracted according to the protocol described earlier (Garcia et al 1990, Howaida & Kemppainen 2003).

Briefly, one ml culture supernatant was taken in a 15 ml polypropylene centrifuge tube and 0.8 ml water was added and vortex mixed. Then, 0.5 ml of 10N sodium hydroxide was added and vortex mixed. To this, 5ml of ethyl ether : n-hexane, 80:20 (v/v) was added and vigorously shaken. The mixture was centrifuged for 5 min at 850 g and the organic layer was separated and dried at room temperature under a stream of nitrogen. The residue was re-dissolved in 1 ml of mobile phase and filtered through a 0.22µm pore sized filter and 20µl was used for HPLC analysis.

B. HPLC analysis
Chemicals and Reagents: Methanol, Water, Ethyl ether, Hexane and Chloroform used were all of HPLC grade and glacial acetic acid, Sodium hydroxide were of analytical reagent grade.
A stock solution of 1 mg/ml levamisole was prepared in methanol. It was stored at -20°C and used.
Chromatography: A HPLC system (Waters, USA) fitted with a 5µm C₁₈ (Octadecylsilane), 150 mm x 4.6 mm analytical column (Phenomenex, USA), guard column packed with Perisorb RP-18 (Upchurch Scientific, USA) and with a UV- detector was used. The chromatography was carried out using the following conditions:
Elution: Isocratic, Sample volume: 20ul, Flow rate: 1ml / min.
Mobile phase: 2% Acetic acid in water, methanol (50:50(v/v)) pH adjusted to 7.30 with 10N NaOH solution.
Detection: U.V. detection at wavelength of 225nm.
Levamisole, 5-20 µg was analyzed as standard along with the extracted samples. The concentration in the samples was determined using a graph obtained with vales of standard levamisole.
2. $^3$H-levamisole binding

Resting & mitogen stimulated murine & human peripheral blood lymphocytes and myeloma cells were used for binding studies.

A. $^3$H-levamisole binding to intact cells

Reagents: Bray’s mixture (0.1 % Triton-X 100, 4gm PPO, 200mg POPOP, 60gm Naphthalene, 20ml ethyleneglycol, 100ml methanol- volume made up to 1 ltr with 1,4-Dioxan).

Procedure: 1 X $10^6$ cells were taken and washed twice with RPMI-1640 and then resuspended in 100µl of RPMI-1640, $^3$H-levamisole (50 nmoles, 1,35,000 cpm) was added and incubated at 37°C for 1hr. At the end of incubation, the cells were washed twice with RPMI-1640 and finally the cell pellet was lysed using 100µl of 0.1% TX-100 and counted in Bray’s mixture.

B. $^3$H-levamisole binding to lysate

Procedure: 1 X $10^6$ cells were taken and washed twice with RPMI-1640 the cell pellet was then lysed with 0.1% Triton X-100. To 0.10 ml of the cell lysate, $^3$H-Levamisole (50 nmoles, 1,35,000 cpm) was added and incubated for 1hr at 37°C. Then 10 % TCA was added to a final concentration of 10% and incubated for 1hr at 4°C. The precipitate was pelleted by centrifugation at 5000g for 10min and the pellet was washed twice with 5% TCA and finally with ether. The pellet was air dried and dissolved 100µl of 0.1% TX-100 and counted in Bray’s mixture (Moreno-Guzman et al 1998).

Non-specific binding was determined in the presence of a 1000-fold molar excess of unlabelled compound. Non-specific binding was subtracted from the total in order to obtain the specific binding.
Results:

3H-levamisole binding

Tritium labeled levamisole binding assays were performed using unstimulated and mitogen stimulated murine splenic lymphocytes and human peripheral blood lymphocytes. Earlier studies have shown that mitogen-stimulated murine splenic lymphocytes show enhanced APase activity. This experimental system was used for comparative purposes to assess the binding of 3H-levamisole to myeloma cell lines.

LPS was used for mitogenic stimulation in case of murine splenic lymphocytes and PWM was used for human peripheral blood lymphocytes. LPS stimulated murine splenic lymphocytes showed enhanced alkaline phosphatase activity (Fig 5.1). No enhancement of APase activity was observed in human peripheral blood lymphocytes upon mitogenic stimulation (Fig 5.2). However, myeloma cell lines displayed significant APase activity (Fig 5.3).

The binding of 3H-levamisole was significantly higher in lysates of LPS stimulated murine splenic lymphocytes as compared to whole cells (Fig 5.4). The binding of 3H-levamisole to human PBL was minimal in whole cells as well as in lysates and there was no difference between unstimulated and PWM stimulated cells (Fig 5.5). The myeloma cells which express APase activity had significant 3H-levamisole binding (Fig 5.6). In all the cases, 3H-levamisole binding correlated well with the expression of APase activity.

Analysis of Levamisole by HPLC

Levamisole eluted with a retention time of about 6.0 minutes on reverse phase column under the conditions employed. Levamisole ranging from 4-20 µg concentration was used (Fig 5.7 A-D) and a standard graph was calibrated (Fig 5.8). The values used to construct standard graph are presented in Table 5.1. Levamisole was extracted from myeloma cell cultures at various time points 0, 24, 48, 72h and analysed by HPLC. The extracts obtained with RPMI and RPMI + FCS was analysed and the elution profiles
Alkaline Phosphatase activity of mitogen stimulated murine splenic lymphocytes

* p < 0.05  control Vs mitogen
Fig. 5.2

Alkaline phosphatase activity of Poke Weed Mitogen stimulated normal human peripheral blood cells

![Bar chart showing Alkaline phosphatase activity for two samples](chart.png)

- Sample 1: No addition
- Sample 2: PWM (2μg)

nmol/L / 0.2 x 10^6 cells
Fig - 5.3

Alkaline phosphatase activity of myeloma cell lines

![Graph showing alkaline phosphatase activity of myeloma cell lines](image)
$^3$H-Levamisole binding of mitogen stimulated murine splenic lymphocytes

1-3: Whole cells  4-6: Cell lysate
* $p < 0.05$ lysate Vs whole cells
Fig - 5.5

$^3$H-Levamisole binding of of Poke Weed Mitogen stimulated normal human peripheral blood cells

1,2 : Whole cells, 3,4 : Cell lysate
* p < 0.05 lysate Vs whole cells
$^3$H-Levamisole binding of myeloma cell lines

![Graph showing $^3$H-Levamisole binding levels for different samples.](image)

- **Sample 1, 2:** Whole cells
- **Sample 3, 4:** Cell lysate

* $p < 0.05$ lysate Vs whole cells
Fig: 5.7 : HPLC Elution Profile of Levamisole.

A. 4µg

B. 8µg
C: 12 µg

D: 20 µg
Standard graph of levamisole

The area under the curve is taken and standard graph is plotted.

Table - 5.1

<table>
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<tr>
<th>Concentration Of Levamisole (µg)</th>
<th>Area under the curve (Arbitrary unit, AU)</th>
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<tbody>
<tr>
<td>4</td>
<td>25399</td>
</tr>
<tr>
<td>8</td>
<td>41837</td>
</tr>
<tr>
<td>12</td>
<td>47257</td>
</tr>
<tr>
<td>20</td>
<td>62950</td>
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are presented in Fig. 5.9 A&B. This was carried out to examine whether any substances present either in RPMI or serum have a retention time similar to that of levamisole. The elution profiles of levamisole at various time periods of culture are presented in the Fig 5.10 A-D, Table 5.2. The elution profiles indicated that compounds with retention times different from that of levamisole were obtained with progressive culture time period. The amount of levamisole recovered from the culture supernatants also decreased with time.
HPLC elution Profile of Levamisole in (A) RPMI 1640 and (B) RPMI + FCS
A. HPLC elution profile of levamisole extracted from culture – ‘0’ time of culture period
B. HPLC elution profile of levamisole extracted from culture – 24 hours of culture period
C. HPLC elution profile of levamisole extracted from culture – 48 hours of culture period
D. HPLC elution profile of levamisole extracted from culture – 72 hours of culture period
Levamisole extracted at various time points and analysed/quantified by HPLC.

<table>
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<th>(h)</th>
<th>RT</th>
<th>AU</th>
<th>(% recovered)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>4.7</td>
<td>40,443</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
<td>22,415</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>5.2</td>
<td>30,382</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>17,414</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>5.7</td>
<td>27,533</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.1</td>
<td>9,966</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>8.3</td>
<td>1,797</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>4.7</td>
<td>15,332</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>5,898</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>1,176</td>
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The values presented are representative of 2 independent experiments.
Discussion:

The results reported in the previous chapters have revealed that levamisole inhibits proliferative response of multiple myeloma cells probably via inhibition of alkaline phosphatase activity. The concentration of the drug required to get half-maximal inhibition in myeloma cells was shown to be around 1mM. This corresponds to a value of 240 µg/ml.

Drugs are transported mostly as complexes with serum albumin. The binding of levamisole to total plasma proteins of 6 animal species was determined in vitro by equilibrium dialysis and it has been shown to bind avidly to plasma proteins. (Sahagun et al 1997). Levamisole being a basic organic drug has weak lipophilic tendency in an alkaline medium (Neilsen & Rasmussen, 1983). It was reported earlier that levamisole when administered to patients with colorectal carcinoma at a concentration of 100mg/m² three times a day, resulted in 1µg/ml of peak plasma concentration (Reid et al 1998). The high concentration required in cell culture could be firstly due to low lipophilic nature of levamisole there by resulting in low binding to membrane proteins and secondly due to binding to serum proteins resulting in lower concentration of free drug available to the cells. It is also possible that the drug undergoes transformation under the culture conditions employed.

In order to address this issue, ³H-levamisole was used to estimate its binding to cells. Earlier, ³H- labeled levamisole specific binding assays revealed a specific binding carrier for levamisole in human peripheral lymphocytes and granulocytes. (Ogawa, 1983). The results from the present study of ³H-levamisole binding assays indicate that the binding was significantly higher in lysates of LPS stimulated murine splenic lymphocytes as compared to whole cells. The binding of ³H-levamisole to human PBL was minimal in whole cells as well as in lysates and there was no difference between unstimulated and PWM stimulated cells. The myeloma cells which express APase activity had significant ³H-levamisole binding. In all the cases, ³H-levamisole binding correlated well with the cells expressing APase activity. Enhanced binding in the lysates compared to whole cells could be due to the exposure of putative levamisole binding site (domain) of APase present on cell membrane facing the cytosol.
Earlier studies have reported the effect of temperature and pH on the chemical stability of levamisole where levamisole was shown to degrade rapidly between pH 5 - 8. (Dickinson et al, 1971 a, b & c). Levamisole solution stored at 4 °C was shown to be stable (Fouad et al 2005). Levamisole stability was assessed when stored at 4 or 37°C and at pH 6, 7, 7.5 and 8. Analysis of the various solutions by high pressure liquid chromatography demonstrated that levamisole degradation occurs during storage in neutral and alkaline conditions to form three products. The formation of the products was accelerated by increasing the temp from 4 to 37°C. The degradation products were purified by preparative high pressure liquid chromatography and their structures determined by spectrometry, IR spectrometry and homo-and heteronuclear two dimensional NMR spectroscopy (Hanson et al, 1991).

Levamisole, used presently in the culture along with cells, medium (RPMI 1640) and fetal calf serum at 37°C up to period of 72h might have affected the stability of levamisole, in order to assess the same HPLC was done. A high-pressure liquid chromatographic with ultraviolet detection methods (HPLC-UV) was used for quantification of levamisole. Calibration curves for levamisole were linear over the range 4-20ug.

HPLC analysis of cell culture supernatants of myeloma cells has shown that products of levamisole appeared with progressive culture period indicating a metabolic transformation. Also the amount of levamisole recovered from the culture supernatants with FCS was lower (less stable) than levamisole extracted from culture samples without FCS. This could be due to additional enzymatic degradation of levamisole in the presence of serum apart from degradation due to temperature. The degradation of levamisole could be one more possible reason for the high concentration of levamisole required to get desirable cytotoxic effect on myeloma cells.