In the Search for New Anticancer Drugs, II
Antitumor Activity, Toxicity and Electron Spin Resonance
of Spin Labeled Thio-TEPA Derivatives

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The spin labeled analog of Thio-Tepa, 1-oxyl-2,2,6,6-tetramethyl-4-piperidyl-
N,N,N',N'-bis (ethylene)-phosphorodiamidotioate (SL-O-TT), which contains a
nitroxyl free radical linked by an oxygen bridge to phosphorus, has antitumor properties
against P388 murine leukemia (T/C = 242) and a higher therapeutic ratio (5.15) than its
parent compound, Thio-TEPA (2.75). The drug is less toxic to P388 cells in culture as
judged by the 3H-thymidine uptake. On the basis of electron spin resonance spectroscopy
using L1210 cells incubated with SL-O-TT, it is concluded that the drug is bound to
cells in culture in such a way as to restrict the motion of the nitroxyl label.

A second spin labeled analog, 1-oxyl-2,2,6,6-tetramethyl-4-amino-piperidyl-
N,N,N',N'-bis (ethylene)-phosphorodiamidothioate (SL-NH-TT), containing a nitroxyl
label linked by a nitrogen bridge to phosphorus, first synthesized by Russian workers,
was prepared by an improved procedure in 95% yield. In vivo results indicate that this
analog has about the same therapeutic value (2.73) as Thio-TEPA (2.75), and that higher
doses of this compound are required than those for both the O-bridged analog and Thio-
TEPA to achieve maximum T/C values.

Introduction

The antitumor activity of Thio-TEPA (Tri-ethyleniminemethiophosphoramid, TT) has been
known since 1963 [1, 2]. In the past, the drug has been used clinically against ovarian carcinoma,
Hodgkin's disease, carcinoma of the breast and other malignancies [3]. In recent years, however, it
has not enjoyed wide-spread clinical application because of its toxicity. Although many synthetic
modifications of Thio-TEPA have been made over the years, no substantial improvement in lowering
the toxicity and raising the activity levels has been achieved.

In the last decade, an approach basically different from the past attempts has been made [4, 5],
whereby one aziridinyl moiety of Thio-TEPA has been replaced by a moiety which not only may
change the biological characteristics of the drug, but also may serve as a marker, such as a nitroxyl
spin label, for monitoring the metabolic pathway of the drug.

Emanuel and coworkers reported [6] that the Thio-TEPA analog containing the NH-bridged spin
label (SL-NH-TT) has a lower toxicity and a higher antitumor activity than thio-TEPA. Since some of
these data [6] were difficult to compare directly with our results, we would now like to report on our
studies concerning firstly, the in vitro cytotoxicity of the oxygen-bridged analog (SL-O-TT) [7] of Thio-
TEPA, as compared to Thio-TEPA (TT), secondly, the comparison of the in vivo antitumor activity of
SL-O-TT and SL-NH-TT with the parent compound TT, and thirdly, the qualitative ESR spectro-
scopy on tumor cells treated with the oxygen-bridged spin labeled drug (Fig. 1). The work pre-
Materials and Methods

Pure Thio-TEPA (TT) was obtained from Lederle Laboratories (Pearl River, N.Y.) and used without further purification. Spin-labeled Thio-TEPA (SL-O-TT) was synthesized as previously described [7]. A colorimetric estimation of alkylating activity was performed by the method of Friedman and Boger [11] to ascertain drug activity. The alkylating activity of SL-O-TT was 0.65 mols of nor-nitrogen mustard standard per mol of spin-labeled drug, while that of TT was 1.6 mols with respect to the same standard. All concentrations used in this work are final concentrations. The SL-NH-TT was prepared in 95% yield by an improvement of the original procedure [4].

Preparation of 1-oxyl-2,2,6,6-tetramethyl-4-amino-piperidyl-N,N,N'-N'-bis-(ethylene)-phosphorodiamidithioate (SL-NH-TT)

To a solution of thiophosphoryl chloride (0.85 g, 5 mmol) in 25 ml anhydrous benzene was added dropwise at 5–10 °C a solution of 2,2,6,6-tetramethyl-4-amino-piperidine-1-oxyl (0.86 g, 5 mmol) and triethylamine (0.55 g, 5.4 mmol) in anhydrous benzene (15 ml). Following the addition, the reaction mixture was stirred at 10 °C for 1 h, at 20–22 °C for 4 h, then cooled once again to 5 °C. A solution of triethylamine (1.20 g, 12 mmol) in benzene (10 ml) was rapidly added, followed by a solution of aziridine (0.47 g, 11 mmol) in 20 ml benzene which was added slowly, maintaining the temperature at 5–8 °C. Following the addition, the reaction mixture was stirred at 10 °C for 4 h, at 20–23 °C for 15 h, then filtered. The filtrate was concentrated to a solid. The solid was purified by chromatography (Adsorption Alumina, Fisher Chemicals), with dichloromethane as the eluant. On removal of the solvent from the red-colored fraction, there was
obtained a solid (1.53 g) which was recrystallized from benzene-hexane (1:3). Recrystallization gave 1.50 g (95%) of 1-oxyl-2,2,6,6-tetramethyl-4-amino-piperidyl-N,N,N',N'-bis(ethylene)-phosphorodi-amidothioate, as pale pink needles, m.p. 136.5 to 137 °C.

**Analysis for C₁₃H₂₃N₄OPS**

Calcd C 49.19 H 8.26 N 17.65, Found C 48.94 H 8.08 N 17.90.

MS [m/e]: 319 (M+ + 2), 318 (M+ + 1), 317 (M+).

L 1210 murine leukemia cells were maintained in vitro by serial culture in RPMI 1630 medium (National Institutes of Health Media Unit, Bethesda, MD) containing 50 units/ml of penicillin, 50 μg/ml of streptomycin, 2 μmol/ml of L-glutamine (Flow Laboratories, Rockville, MD) and 15% fetal bovine serum (FBS, Flow Laboratories). Under these conditions, cells had a population doubling time of 12 to 14 h, and achieved a maximum density of 1.5 to 2.0 × 10⁶ cells/ml. P 388 murine leukemia cells were prepared solution of SL-O-TT gave a g value of 2.0028 in the reference cavity. Strong pitch at g = 2.006 and a nitrogen coupling of aN=16.9 Gauss (Fig. 2 A). At low modulation amplitudes, i.e., 0.05–0.1 Gauss, one can see hyperfine lines of approximately 0.5 Gauss, presumably due to interaction of the unpaired electron with the methyl groups [14].

Control animals consisted of mice which were inoculated with tumor cells but not treated, and healthy or tumor bearing mice which were injected daily for 10 days either with saline or with saline containing 3–5% ethanol. Additional in vivo “ethanol controls” consisted of a parallel experiment during which Thio-TEPA was administered at the same ethanol concentrations as required for its analog. The effect of ethanol on the T/C values was found to be insignificant.

**Results and Discussion**

Analysis of the ESR spectrum of a freshly prepared solution of SL-O-TT gave a g value of g = 2.006 and a nitrogen coupling of aN = 16.9 Gauss (Fig. 2A). At low modulation amplitudes, i.e., 0.05–0.1 Gauss, one can see hyperfine lines of approximately 0.5 Gauss, presumably due to interaction of the unpaired electron with the methyl groups [14].

When 3.8 × 10⁻⁷ M SL-O-TT drug is incubated with L 1210 cells (2 × 10⁶ cells/ml) for 2 h, and the cells washed, as described in Materials and Methods, one obtains the ESR spectra shown in Figs. 2B and D. These spectra represent a nitroxyl free radical undergoing a hindered rotation as judged from the broadening of the lines and the decreased intensity of the high field line. The implication is that the drug was involved in an alkylation reaction, and is now bound to the cell in such a way as to hinder the motion of the nitroxyl radical. The spectra shown in Figs. 3B and C are those of the sediment and supernatant, respectively, of
sonicated cells centrifuged at 700 x g in a table-top centrifuge for 20 min. The spectrum of the sediment, which is resuspended in a volume of saline equal to that of the supernatant, is identical to the spectrum of the drug in whole cells (Fig. 3A), while the spectrum of the supernatant is that of a freely rotating drug.

When drug concentrations are decreased to approximately 1 x 10^-5 to 5 x 10^-5 M, only traces of SL-O-TT are detectable, perhaps because at these concentrations, a metabolic reduction of the nitroxy radical to nonradical species [15] lowers the concentration of free radicals beyond detection. Biochemical reduction of the label always occurs, but the rate of reduction appears to vary. The spectra in Fig. 2 can be obtained from cells in log or lag phase at drug concentrations of 3.8 x 10^-4 M or higher. However, cells in log phase metabolically reduce the label considerably more than those in lag phase. Attempts at lysing, dissolving the cell membranes, or sonicing the cells and oxidizing the reduced label back to its free radical form were unsuccessful when oxidizing agents such as potassium ferricyanide [16] and/or a mixture of 30% hydrogen peroxide, 1 N potassium hydroxide and water (2:1:1 by volume) [6] were used. In both cases, irreproducibility was a great problem. However, in aqueous solutions, in the absence of cells, SL-O-TT reduced with ascorbic acid can be reoxidized up to 90-95% of its original concentration by a 0.01-0.1 mM solution of potassium ferricyanide. Concentrations of ferricyanide larger than 1 mM tend to broaden the lines beyond detection.
On the basis of this series of experiments, it is concluded that SL-O-TT binds to the cells as expected, and that the drug is found both in the membranes and in the cytoplasm. It is conceivable that the drug may be embedded deeply in the membrane, since when the membranes are solubilized, the mobility of the spin label is increased (Fig. 3D). Some basic questions remain to be answered, such as, whether the label remains attached to the parent compound and if so, whether the detection of the freely rotating label in the supernatant solution of sonicated cells reflects the presence of the free drug. In addition, it would be of interest to determine the origin of the signal in the sediment of sonicated cells (Fig. 3C). These questions can be answered, in part, by cell fractionation which could lead to a correlation of the presence of the label to the alkylating activity [11]. Subsequent research will deal with these problems. In addition, we will study the metabolic reduction rate(s) of the drug's label, using quantitative ESR techniques in conjunction with methods for the reoxidation of the reduced label.

Both Thio-TEPA and SL-O-TT inhibit the incorporation of \(^3^H\)-thymidine by P 388 cells. The concentration of drug required to inhibit the incorporation of the nucleoside by 50% (IC\(_{50}\)), is \(2.1 \times 10^{-4} \text{ M} \pm 0.5\) (mean \(\pm\) SD) for Thio-TEPA and \(6.1 \times 10^{-4} \text{ M} \pm 1.42\) for SL-O-TT. The IC\(_{50}\) value for the spin labeled compound is approximately three times that of Thio-TEPA, reflecting in part, the absence of one aziridine group. In a separate experiment using Chinese hamster ovary (CHO) cells and a cell colony assay, the dose required to achieve a given level of toxicity with both drugs was 2–2.5 times higher for the spin labeled drug [17]. This result correlates well with our in vivo results. At the concentrations of ethanol used in these experiments (0.1–0.3%), no significant effects were observed in the cells’ ability to incorporate \(^3^H\)-thymidine.

The in vivo antitumor effects of Thio-TEPA, SL-O-TT, SL-NH-TT and the nitroxy TANOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) are shown in Table I. Two important results can be derived from these data. Firstly, that both SL-O-TT

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**Table I. Survival of P 388 inoculated mice treated with Thio-TEPA, spin labeled Thio-TEPA analogs and TANOL.**

<table>
<thead>
<tr>
<th>Dose [mg/kg]</th>
<th>Molarity [mM]</th>
<th>Mean Survival time T/C [%]</th>
<th>ILS [%]</th>
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Spin labeled Thio-TEPA

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<td>168</td>
<td>110</td>
<td>68</td>
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<td>2.4</td>
<td>1.5</td>
<td>1.5</td>
<td>138</td>
<td>115</td>
<td>38</td>
<td>15</td>
<td>0/6</td>
</tr>
<tr>
<td>1.3</td>
<td>0.8</td>
<td>0.8</td>
<td>103</td>
<td>112</td>
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<td>12</td>
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<tr>
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<td>0.5</td>
<td>96</td>
<td>99</td>
<td>-4</td>
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and SL-NH-TT have antitumor activity with maximum T/C values at different doses, namely, 13.24 mg/kg for SL-O-TT and 23.54 mg/kg for SL-NH-TT. Secondly, that the maximum T/C value observed with SL-NH-TT (206) is achieved at a dose (23.5 mg/kg) approximately 3 times higher than that for a similar value of 218 (at 7.5 mg/kg) for SL-O-TT and 8 times higher than that for a value of 213 for TT (3.19 mg/kg). In general, the dose required for similar T/C values for the drugs studied is in the following order: SL-NH-TT > SL-O-TT > TT. Thus, the decrease in toxicity of the labeled compounds is coupled to the requirement for a higher dose to achieve maximum T/C values. The decrease in toxicity can be attributed, in part, to the presence of one less aziridine group in SL-O-TT and SL-NH-TT as compared to TT, and to the nature of the bridge, i.e., O or NH, by which the label is attached to phosphorus. In the present work, the NH-bridged compound (SL-NH-TT) was found to be less toxic and less effective in vivo than the O-bridged compound (SL-O-TT). The advantage of using the spin labeled compounds may lie in the fact that they are active over a broader dose range than the parent TT. Therapeutic index values were calculated (Table I) as previously described [13], and reflect, the broader activity range for SL-O-TT. Thus, the therapeutic index for SL-O-TT (5.15) is approximately twice that for SL-NH-TT (2.73) and TT (2.75). We chose to use TANOL to evaluate the possible contribution of the nitroxyl group to the drug’s activity. Our results indicate that TANOL has no effect of its own. The doses which were used with this nitroxyl were, in general, higher than those used with SL-O-TT or SL-NH-TT, yet even at these higher doses, TANOL had no activity. At the highest dose used, (441 mg/kg), the animals convulse for about one hour and then return to normal. Nevertheless, this result does not necessarily mean that the nitroxyl moiety in the anticancer drug has no beneficial effect [6].

Conclusions

The results of our work using P 388 leukemia are in general agreement with those of Emanuel and coworkers [6] on the decreased toxicity of SL-NH-TT. However, no higher antitumor activity of SL-NH-TT as compared to TT is found against P 388 leukemia, unlike what was found by Emanuel and coworkers against other tumors [6]. On the basis of ESR experiments, it is evident that the label is rapidly reduced by cells in culture [18-20], probably to the corresponding hydroxylamine derivative [18]. In order to use these compounds for pharmacokinetic purposes, it is essential to develop a technique for the reoxidation of the reduced label, ideally to >95% of its original concentration.

One of us (G. S.) would like to thank the National Foundation for Cancer Research for their support of this work.