In the Search for New Anticancer Drugs, II¹

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)-phosphorodiamidothioate (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-ethyleneiminethiophosphoramidate, TT) has been known since 1953 [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whereby one aziridinyl moiety of Thio-TEPA has been replaced by a moiety which not only may change the biological characteristics of the drug, but also may serve as a marker, such as a nitroxyl spin label, for monitoring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 spectroscopy on tumor cells treated with the oxygen-bridged spin labeled drug (Fig. 1). The work pre-

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Fig. 1. Structures of Thio-TEPA, Spin-Labeled Drugs SL-O-TT and SL-NH-TT, and TANOL.

sented here is part of a long-range project in designing antitumor drugs containing nitroxyl labels to be studied for their antitumor potential and possible application in pharmacology and pharmacokinetics.

The field of spin labeling has been expanding considerably in recent years [8–10]. In principle, the method consists of introducing a stable paramagnetic species, generally a nitroxyl moiety, into a biological or chemical system, and using ESR spectroscopy for the detection of these reporter groups. The evaluation of the ESR spectra yields information on the environment in which the spin labeled molecule is located. Nitroxyls are long-lived free radicals. The relatively low reactivity of nitroxyl radicals as compared to other long-lived radicals is attributed to the delocalized nature of the N–O bond, $>\dot{\text{N}}-\text{O} \leftrightarrow \text{N}^+=\text{O}^-$, the steric hindrance caused by the bulky alkyl, usually methyl, groups in the $\alpha, \alpha, \alpha', \alpha'$ -positions, and the inability of these $\alpha, \alpha, \alpha', \alpha'$ -tetraalkyl substituted nitroxyl radicals to undergo a disproportionation reaction to nitron and hydroxylamine derivatives, which is possible in the case of nitroxyl radicals containing α -hydrogens [10]. Unfortunately, there is also a drawback in using the nitroxyl radicals as reporter moieties, since they can readily undergo reductive reactions to the corresponding diamagnetic hydroxylamine derivatives [8] which cannot be detected by ESR. As a result, an oxidative treatment of reduced radicals is essential in order to apply the ESR techniques to such systems.

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)-phosphorodiamidothioate (SL-NH-TT)

To a solution of thiophosphorylchloride (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-aminopiperidyl-N,N;N',N'-bis(ethylene)-phosphorodiamidothioate, as pale pink needles, m.p. 136.5 to 137 °C.

Analysis for $C_{13}H_{26}N_4OPS$

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 2.0×10^6 cells/ml. P 388 murine leukemia cells were maintained *in vitro* by serial culture in a 1:1 mixture of RPMI 1630 and RPMI 1640 media containing 0.01 mM mercaptoethanol, 15% FBS, and penicillin, streptomycin, and L-glutamine as in the above medium. Under these conditions, the cells had a population doubling time of 14 to 18 h and achieved maximum cell densities of 2 to 2.5×10^6 cells/ml. All experiments with L 1210 or P 388 cells were performed in their respective media. Cell counts were obtained on a ZB model Coulter counter.

Incorporation of radioactive thymidine was performed as follows: after incubation of 10^6 cells/ml of P 388 for 120 min with Thio-TEPA or SL-O-TT, 0.1 ml of medium containing 1 µCi of methyl- 3H thymidine (2.0 Ci/mmol, New England Nuclear, Boston, MA) was added to a 1 ml final volume of culture. After further incubation for 60 min with the radiolabeled nucleoside, the cells were assayed for incorporation of 3H into material which was precipitated with trichloroacetic acid as described by Felsted *et al.* [12].

ESR spectra were obtained with a Varian E-109 Century Series Spectrometer using a rectangular TE-104 dual cavity with a strong pitch standard in the reference cavity. Strong pitch at $g = 2.0028$ was used to evaluate experimental g -values. For ESR measurements, cells of L 1210 were incubated for 2 h with SL-O-TT, and then washed four times with cold normal saline. After the last wash, the cells were resuspended in saline at a final concentration of 1.7×10^7 cells/ml and placed in an ESR cell suitable for measurement of aqueous solutions. The supernatant of the fourth wash did not contain any material to cause a detectable ESR signal. Cell viability was 90–95% for all experiments.

Antitumor activity *in vivo* was evaluated against P 388 murine leukemia by the protocols of the National Cancer Institute [13]. Approximately 10^5 cells

were injected i.p. into CDF1 mice at day 0 of the experiment (6 mice/group). At day 1, daily i.p. drug injections began (5.0 ml/kg weight) and continued for 10 days, in the concentrations listed in Table I. The animals were then observed for 30 days keeping a record of deaths and survivors. Data were analyzed by the T/C method [13] where T represents the mean survival time of the treated group, and C the mean survival time of the tumor bearing control group. The percent of increase life span (ILS) parameter was evaluated by $[T-C]/C \times 100$. Therapeutic index is defined as the ratio of the drug dose at maximum percent ILS to that at 40% ILS [13].

Because of their lower solubility, the spin labeled drugs were first dissolved in ethanol, and then saline was added to achieve the required drug concentrations. The maximum amount of ethanol used *in vivo* was approximately 3–5%, necessary for the highest drug concentration. This amount of ethanol was reduced accordingly as lower drug concentrations were used. For *in vitro* experiments, the ethanol required was 0.1–0.3%.

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 $a_N = 16.9$ 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^{-4} M SL-O-TT drug is incubated with L 1210 cells (2×10^6 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

Fig. 2. 9.3 GHz electron spin resonance spectra of: (A) Spin labeled Thio-TEPA (SL-O-TT) (5.6×10^{-4} M). (B) L 1210 cells incubated in 5.6×10^{-4} M spin labeled Thio-TEPA (SL-O-TT) for 2 h at 37°C , 5% CO_2 and 95% humidity followed by 4 rinses with cold normal saline; (C) fourth rinse supernatant; (D) similar to spectrum B, except for a wider magnetic field range and signal averaging for 160 min using a Nicolet 1180 computer. The magnetic field increases from left to right. The ESR conditions at room temperature were: 8 mW incident microwave power, 1 Gauss modulation amplitude. Relative gains were as shown in the Figure. Strong pitch at $g = 2.0028$ was used as a standard.

sonicated cells centrifuged at $700 \times g$ 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 1×10^{-5} to 5×10^{-5} M, only traces of SL-O-TT are detectable, perhaps because at these concentrations, a metabolic reduction of the nitroxyl radical to nonradical species [15] lowers the concentration of free radicals beyond detection. Biochemical reduction of the label always occurs, but

Fig. 3. 9.3 GHz spectra of: (A) L 1210 cells incubated for 2 h with 5.6×10^{-4} M spin labeled Thio-TEPA (SL-O-TT) at 37°C , 95% humidity and 5% CO_2 ; (B) sediment of sonicated cells previously incubated with drug and resuspended in a volume of saline equal to that of the supernatant; (C) supernatant of sonicated cells previously incubated with drug; (D) incubated cells treated with the non-ionic detergent triton (Sigma X-100). The ESR conditions at room temperature were: 8 mW incident microwave power, 1 gauss modulation amplitude. Relative receiver gains were as shown in the Figure. Strong pitch at $g = 2.0028$ was used as a standard. The magnetic field increases from left to right.

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 3.8×10^{-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 sonicating 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 re-oxidized 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 ^3H -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 ^3H -thymidine.

The *in vivo* antitumor effects of Thio-TEPA, SL-O-TT, SL-NH-TT and the nitroxyl 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

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

	Dose [mg/kg]	Molarity [mM]	Mean Survival time T/C [%]	ILS [%]	Survivors 30 days			
Thio-TEPA	7.55	8.00	91	-8	0/6			
	5.66	6.00	253	153	3/6			
	4.25	4.50	239	139	2/6			
	3.19	3.38	213	113	0/6			
	1.79	1.90	125	25	0/6			
	1.01	1.07	118	18	0/6			
	0.57	0.60	113	13	0/6			
Spin label (TANOL)	441.00	500.00	105	5	0/4			
	221.00	250.00	101	8	0/4			
	111.00	125.00	95	-6	0/4			
	55.00	62.50	103	3	0/4			
Spin labeled Thio-TEPA								
	SL-O-TT	SL-NH-TT	SL-O-TT	SL-NH-TT	SL-O-TT	SL-NH-TT	SL-O-TT	SL-NH-TT
41.9	26.3	26.4	95	195	-6	95	0/6	0/6
23.5	14.8	14.9	108	206	8	105	0/6	0/6
17.7	11.1	11.2	235	191	135	91	5/6	0/6
13.2	8.3	8.4	242	160	142	60	4/6	0/6
9.9	6.3	6.3	231	142	131	42	2/6	0/6
7.5	4.7	4.7	218	143	118	43	0/6	0/6
5.6	3.6	3.5	178	124	78	24	0/6	0/6
4.2	2.6	2.7	168	110	68	10	0/6	0/6
2.4	1.5	1.5	138	115	38	15	0/6	0/6
1.3	0.8	0.8	103	112	3	12	0/6	0/6
0.7	0.5	0.5	96	99	-4	-1	0/6	0/6

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 eval-

uate 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.

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