Cancer Chemother Pharmacol (1985) 15: 185–191

Cancer Chemotherapy and Pharmacology

Original articles

On the search for new anticancer drugs 14: The plasma pharmacokinetics and tissue distribution of spin-labeled thio-TEPA (SL-O-TT)*

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Summary. We defined the plasma and tissue concentrations and pharmacokinetics of SL-O-TT, a spin-labeled analog of thio-TEPA, in 35–44-g male Swiss Wesbster mice that had received spin-labeled thio-TEPA at a dosage of 10 mg/kg. Concentrations of spin-labeled thio-TEPA in ethyl acetate extracts of tissue and plasma were determined by gas-liquid chromatography and electron spin resonance spectroscopy. Plasma concentrations of spin-labeled thio-TEPA declined in a biexponential fashion that was well described by the equation:

$$C_t = 21.5e^{-0.276t} + 2.30e^{-0.026t}$$

indicating a half-life alpha of 2.5 min and a half-life beta of 26.6 min. After 2 h there was still spin-labeled thio-TEPA in plasma, but not in tissues. In tissues, no spin-labeled thio-TEPA was detected with gas-liquid chromatography 15 min after injection, but with electron-spin resonance label was found in lung and skeletal muscle. The main metabolite of spin-labeled thio-TEPA is spin-labeled TEPA, where oxidative desulfurization is invoked as the main metabolic mechanism. Reduction of the spin label to the hydroxylamine was also observed with time.

Introduction

Thio-TEPA (*N,N',N''*-triethylene thiophosphoramide) is an alkylating agent that belongs to a family of compounds containing pentavalent phosphorus and the aziridine moiety (Fig. 1). The activity of these compounds has been known for many years [2, 9, 12, 17, 20, 22, 23]. Thio-TEPA is presently used in the chemotherapy of metastatic carcinoma of the breast [9, 17], superficial carcinoma of the bladder [12, 16, 22], and carcinomatous meningitis [8, 21]. We are interested in the potential of thio-TEPA analogues as antitumor agents, and have investigated the activity of seleno TEPA [13] and of spin-labeled derivatives of thio-TEPA [7]. In seleno TEPA, the sulfur atom of thio-TEPA is substituted by a selenium atom. Seleno TEPA was found to be less active than thio-TEPA for cells in vitro [13]. Spin-labeled derivatives of thio-TEPA [5, 7] are compounds in which one of the aziridines is replaced by a nitroxyl (spin label) free radical. For instance, SL-O-TT contains a tetramethylpiperidine nitroxyl free radical linked by an oxygen bridge to pentavalent phosphorus (Fig. 1). Nitroxyls are stable free radical moities (Fig. 1) which give a distinct electron spin resonance (ESR) spectrum consisting of three ESR lines due to the coupling of an unpaired electron to a nitrogen nucleus. The original rationale for designing an antitumor agent with a nitroxyl moiety was that the biological activity would be changed (e.g., reduced toxicity), the label would be used as a tracer (e.g., pharmacokinetics, drug metabolic pathway), and there had been a report that stable free radicals such as nitroxyls had antitumor activity [15]. We have investigated some of the effects which the introduction of the spin label has on thio-TEPA in terms of biological activity. Thus, SL-O-TT and SL-NH-TT (Fig. 1) were evaluated in vivo against P388 murine leukemia [7]. We found that the dose required to reach similar activity (i.e., similar treated survival/control survival values) were in the following order SL-NH-TT > SL-O-TT > thio-TEPA. On the basis of ESR, we found that SL-O-TT binds to nuclei, microsomes, and mitochondria of L1210 cells incubated for 1 h with the drug [14].

In this paper we continue to study SL-O-TT by defining the plasma pharmacokinetics and tissue distribution of the drug in mice after an IV bolus injection. We have recently reported a study of this kind for the parent compound thio-TEPA, in which gas-liquid chromatography (GLC) was used [3, 6]. In this paper, we report not only the use of GLC to detect SL-O-TT, but also the use of ESR spectroscopy. With GLC, SL-O-TT is detected with a nitrogen-phosphorus detector, whereas with ESR, the spin label moiety is detected.

Materials and methods

Adult, male Swiss Webster mice were obtained from Microbiological Associates (Walkersville, Md). Animals were housed with an alternating 12-h light 12-h dark schedule, and were given food and water ad libitum. Animals 12–16 weeks old and weighing 35–44 g were used for this study. Spin-labeled thio-TEPA (1-oxyl-2,2,6,6-tetramethyl-4-piperidyl-*N,N:N',N'*-di-1,2-ethanediylphosphorodiamidothioate) (SL-O-TT) was synthetized as previously described [18]. SL-O-TT was first dissolved in ethanol, then

* This work was supported by a grant from the Lederle Laboratories, and by a grant from the Bressler Foundation. The synthesis of spin labeled compounds was supported by a grant from the National Foundation for Cancer Research to GS
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diluted with 0.154 *M* sodium chloride to give a final concentration of 2 mg/ml and 1% ethanol. The drug was administered at a dosage of 10 mg/kg as an IV bolus via the tail vein. The volume injected was 0.20–0.30 ml per mouse.

At 1, 3, 5, 10, 15, 30, and 60 min, and 2, 4, 6, 12, 16, and 24 h after injection, groups of three mice were anesthetized with diethyl ether, and blood samples were obtained from the retro-orbital venous plexus. Blood was collected in Eppendorf microcentrifuge tubes (Brinkman Instruments, Inc., Westbury, NY.) each containing 50 units of heparin per tube, and placed on ice. Animals were then killed by cervical dislocation and rapidly dissected. Brains, hearts, lungs, livers, kidneys, spleens, and skeletal muscles were weighed, immediately frozen on dry ice and stored at −30 °C until analyzed. Blood samples were centrifuged at 12 000 *g* at 4 °C for 2 min. The resulting plasma supernatant was frozen on dry ice and stored at −30 °C. All samples were analyzed within 7 days.

The spin-labeled analogue of TEPA (SL-O-T)(1-oxyl-2,2,6,6-tetramethyl-4-piperidyl-*N,N:N',N'*-di-1,2-ethanediylphosphorodiamidate) (Fig. 1) was synthesized as previously described [18] and used in experiments aimed at identifying SL-O-TT metabolites.

Sample extraction and analysis. Tissue samples were mixed with four volumes of cold ethyl acetate containing a known concentration of *P*-nitrophenylacetonitrile (PNPAN) (5 or 50 μg/ml) as an internal standard, and were homogenized in a ground glass mortar and pestle homogenizer. Extracts were centrifuged at 12 000 *g* at 4 °C for 5 min. The supernatant (1 μl) was injected into a Hewlett-Packard model 5840A gas chromatograph. The rest of the supernatant was analyzed directly by ESR as described below, or blown dry under a nitrogen stream and stored. In some cases, samples were extracted without PNPAN for ESR measurements. The gas chromatograph was fitted with a 1.8 m × 2 mm (internal diameter) glass column packed with 3% OV-225 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, Pa) [6]. The oven and injector were maintained at 210 °C and 230 °C, respectively. Nitrogen, at a rate of 30 ml per min, was used as the carrier gas. Detection was accomplished with a nitrogen-phosphorus detector, maintained at 230°C with an air flow rate of 100 ml/min, a hydrogen flow rate of 7 ml/min, and a bead voltage of 18–20 V. Peaks were traced and integrated with as low as Hewlett-Packard 5840A GLC terminal, and concentrations of SL-O-TT were determined by comparison with peak areas of the PNPAN internal standard. This procedure allowed measurement of plasma and tissue concentrations of SL-O-TT as low as 0.02 μg/ml with a precision of 1%.

Control experiments, in which known concentrations of SL-O-TT were extracted from triplicate samples of plasma or 1 : 3 homogenates of tissue in 0.154 *M* NaCl, demonstrated an extraction efficiency of greater that 81% from plasma and each tissue studied. Plasma did not contain endogenous materials that interfered with the determination of SL-O-TT or internal standard, but brain and kidney did.

The ESR analysis was performed in a Varian E109 Century Series spectrometer (Varian Instruments, Palo Alto, Calif) that had a rectangular TE104 dual cavity with a spin label standard (2,2,6,6-tetramethylpiperidinoxyl, TEMPO), (Aldrich Chemical Co., Milwaukee, Wis) in the reference cavity and the sample in the front cavity. Dried samples previously used for gas chromatography determinations or extracted and dried for ESR determinations were resuspended in 20 μl methanol, vortexed, allowed to fill a 20-μl pipette (Drummond Microcaps) by capillary action, and sealed at both ends with seal-ease clay (Clay Adams, Parsippany, NJ). The samples were subsequently placed inside a Pasteur pipette sealed at one end and placed in the front cavity of the ESR spectrometer for measurements. The capillaries were filled with sample (3 cm) to cover the sensitive region of the cavity. Standard samples were also placed in 20-μl capillaries, inserted into sealed Pasteur pipettes, and placed in the spectrometer's back cavity. The same sealed Pasteur pipettes were used for sample and standard throughout the experiment. Exchange of standard and experimental samples in the dual cavity allowed for calculating a relative sensitivity factor evaluated as the ratio of the signal strength of the standard in the front cavity to that in the back cavity. The concentration of SL-O-TT (measured by the concentration of spin label) in plasma and other tissues was evaluated by double integration of the observed spin label signal from SL-O-TT and the external standard. The areas under the absorption curve for samples were related to the areas of the standard of known concentration as described by Hyde [11], but taking into account extraction efficiency, appropriate dilutions, and the fact that the molecular weight of SL-O-TT is approximately twice that of the spin label TEMPO. Identical results were obtained when SL-O-TT was used as a standard. TEMPO was used as a standard in this case because it is available commercially and SL-O-TT is difficult to synthesize. The presence of the PNPAN standard used in the GLC assay had no effect on the spin label. Because the signals were at times very weak, it was necessary to average the signal for up to 3 h with the aid of a Nicolet 1180 computer (Nicolet Instrument Co., Madison, Wis) interfaced with the ESR spectrometer. Double integrals were evaluated with the Nicolet 1180 computer with an accuracy of ±15%. Because nitroxyl free radicals are biochemically reduced to the corresponding hydroxylamine, an attempt was made to reoxidize the samples with lead oxide suspended in benzene. (P. L. Gutierrez et al., 1985, unpublished results). This reagent is milder than other oxidants (e.g. ferricynide), which tend to break down the molecule and form several products (P. L. Gutierrez et al., 1985, unpublished results). Because the samples in this case were so small and difficult to obtain (0.4 ml at the most), and because in some trials the results were not uniform, we decided to proceed with the ESR pharmacokinetic assay of SL-O-TT without reoxidation. In addition, as the experiment began, we observed a metabolite by GLC. Because at that time we did not know the identity of the metabolite it was decided not to oxidize the samples, to avoid unforeseen complications.

Pharmacokinetic analysis. Plasma pharmacokinetics were analyzed with MLAB (an online modeling laboratory, Division of Computer Resources and Technology, National Institutes of Health, Bethesda, Md). Curves were fitted to the sum of two exponentials by a nonlinear regression technique. Concentration times time values ($C \times t$) for the 0–60 min portions of plasma and tissue curves were estimated by calculating the area under the curve (AUC) by means of the trapezoidal rule.

Results

Plasma pharmacokinetics

Gas liquid chromatography. The GLC chromatograph of SL-O-TT in our conditions gave two peaks with retention times (RT) at 2.4 min (peak a') and 6.1 min (peak a) (Fig. 2 and 3). The identity of peak a' was suspected to be the reduced form of SL-O-TT (Fig. 1), where the nitroxyl free radical becomes the diamagnetic hydroxylamine. This suspicion was confirmed by reducing SL-O-TT with ascorbic acid (2:1 ascorbic acid to SL-O-TT) [19] and observing a

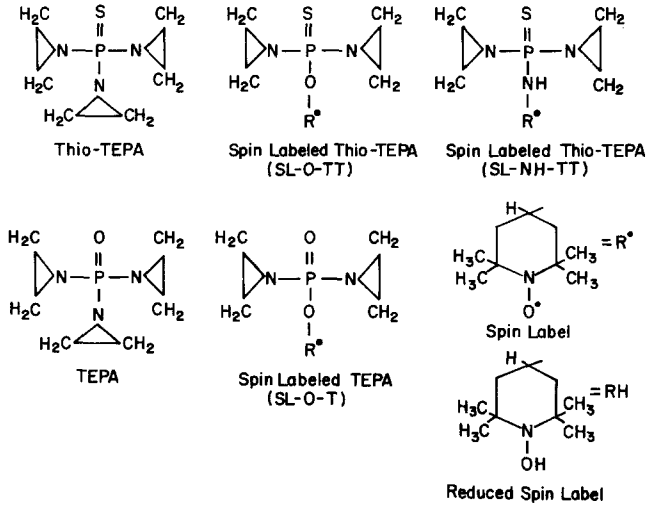

Fig. 1. Chemical structures of thio-TEPA and spin-labeled analogues

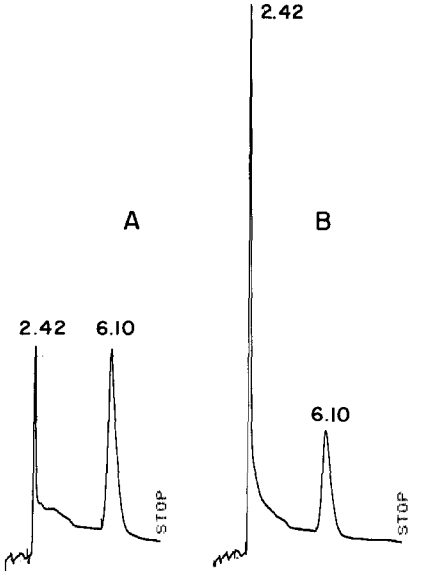

Fig. 2. GLC chromatogram of SL-O-TT (3.18 mg/ml) before **(A)** and after **(B)** reduction with ascorbic acid (2:1 ascorbic acid to SL-O-TT). SL-O-TT was incubated in methanol with ascorbic acid for 10 min, dried under a nitrogen stream, resuspended in 50 µl ethyl acetate and injected into a Hewlett-Packard 5480A chromatograph. The GLC conditions for the chromatograph packed with 3% OV-225 on 100/120 Suplelcoport were: 250 °C for injector and N-P FID detector, 230 °C for the oven, 30 ml/min nitrogen flow rate, and 100 and 7 ml/min for air and hydrogen flow rates, respectively. The head voltage was set between 18 and 20 V.

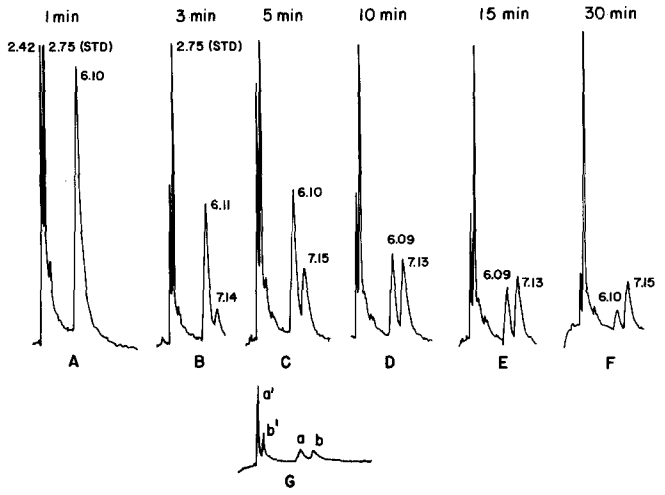

Fig. 3. GLC chromatograms of plasma extracts from mice taken at various times after an IV, bolus injection of SL-O-TT (10 mg/kg). Chromatogram *G* is from a dilute sample of 10-min plasma without internal standard, which was kept frozen for 3 months. The GLC conditions were the same as in Fig. 2, except that the injector temperature was 210 °C and the detector 230 °C for chromatograms *A–F*. The peak at RT 6.1 min represents SL-O-TT, and the peak at Rt 7.1 min represents its metabolite SL-O-T

drastic increase in peak a' with a concomitant decrease in peak a. In the case of Fig. 2, before reduction, 20% of the total concentration was due to peak a'. After reduction, this concentration increased to 292%. GLC chromatograms of fresh SL-O-TT always showed these two peaks in different proportions (5%≤) depending on the preparation, but the ratio of the peaks always remained constant within a given preparation. This allowed for accurate determination of SL-O-TT concentration in plasma and tissues. When SL-O-TT was chemically (Fig. 2) or biologically reduced (e.g., Fig. 3G), peak a' would be large and a concomitant decrease of peak a (the parent compound) would be observed. Because reduced SL-O-TT is not an expected contaminant of SL-O-TT (P. L. Gutierrez et al., 1985, unpublished result), we believe that this peak arises from possible thermal rearrangement in the GLC column, and that an unidentified contaminant may be involved as a precursor.

In plasma samples, the decline of SL-O-TT concentrations and the increase of a metabolite with RT 7.1 min were observed with time (Fig. 3 and 4). Plasma concentrations of SL-O-TT declinded in a biexponential fashion which was well described by the equation:

$$C_t = 21.5e^{-0.276t} + 2.30e^{-0.026t}$$

where C_t represents the concentration of SL-O-TT present at any given time (t) (Fig. 4). Therefore, IV-injected SL-O-TT has a very rapid distributive phase with a half-life ($t_{1/2}$) of 2.5 min, and a somewhat longer terminal half-life of 26.6 min. The peak plasma concentrations of SL-O-TT measured 1 min after drug injection was 20.4 ± 1.02 µg/ml (mean ±SEM). By 2 h after the injection SL-O-TT concentrations could still be measured, but by 4 h the absolute SL-O-TT concentration present was below the quantitative limits of our procedure, even though the presence of SL-O-TT can be demonstrated by a small peak on the chromatographic tracing. The area under the curve (AUC) of plasma concentrations of SL-O-TT from zero to 2 h is 166.8 µg

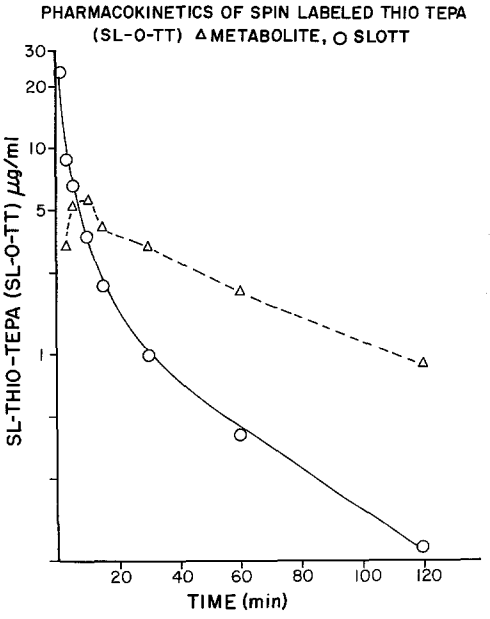

Fig. 4. Pharmacokinetics of SL-O-TT in plasma measured by GLC. *Circles* represent concentration of SL-O-TT in plasma of mice that had received 10 mg/kg SL-O-TT. *Points* represent the means of determinations for three mice/time (Table 1). *Triangles* represent the SL-O-TT metabolite SL-O-T (see Fig. 3). The *solid line* represents a fit to a biexponential curve.

ml/min. For this calculated value and dose injected, the total body clearance (CL_{TB}) of SL-O-TT can be calculated from the equation $CL_{TB} = \frac{\text{dose}}{\text{AUC}}$ to be 59.9 ml/min/kg. The apparent steady-state volume of distribution of the drug is 53.6 ml.

The concentrations of the metabolite at RT 7.1 min increased with time, reaching a maximum near 10 min after injection. Thereafter, the concentrations declined but the metabolite was always present in larger concentrations than the parent compound (Fig. 4).

Electron Spin Resonance Spectrometry. The plasma concentrations of SL-O-TT measured by ESR declined exponentially, and by 60 min after injection the concentrations had reached levels below the sensitivity of the instrument (Table 1). It is interesting to note that the concentrations detected by ESR correspond closely to the total concentrations of SL-O-TT plus its metabolite detected by GLC. This is because ESR measures the concentration of the total nitroxyl free radical labels and is thus not able to discriminate any changes that may have taken place in the rest of the molecule. GLC measurements indicate that by 30 min after the injection at the latest, SL-O-TT was not appreciably reduced to the hydroxylamine, because peak a' decreased at the same rate that the main peak a decreased (e.g., Fig. 3 A–F). In contrast, Fig. 3 G shows a 10-min sample extracted 3 months after the experiment, in which the a' and b' peaks appeared greatly increased. This sample showed no detectable ESR signal.

Identification of metabolite

Craig et al. [22], reported in 1959 that thio-TEPA was metabolized to TEPA (Fig. 1), in rat, dog and rabbit but not in mice. In mice, the principal metabolite of thio-TEPA is organic phosphate with no traces of TEPA [1]. In our systematic search for metabolites, we concentrated primarily on identifying the metabolite observed at 7.1 min retention time (peak b) shown in Fig. 3 A–F. A chromatogram of plasma (approx. 10 min after injection) at low concentration and without an internal standard revealed that an additional peak near 3.3 min was also present (b') (Fig. 3 G). Neither the nitroxyl free radical alone (Fig. 1) nor thio-TEPA enhanced the b' and b peaks as spin-labeled TEPA (SL-O-T) did (Figs. 1 and 5 B). When spin-labeled TEPA was added to plasma extracted as previously described for SL-O-TT, and analyzed by GLC, the peaks sought after (b' and b) were obtained. The b' peak corresponds to the reduced SL-O-T as demonstrated by a similar experiment depicted in Fig. 2, where SL-O-TT was reduced by ascorbic acid. Cochromatography of both SL-O-TT and SL-O-T showed a marked increase of peaks b' and b (Fig. 5 C). This result is consistent with the assignment of the plasma metabolite to spin-labeled TEPA. Plasma concentration levels determined by ESR represent primarily the presence of both SL-O-TT and SL-O-T.

Drug tissue distribution

Gas Liquid Chromatography. In the tissues where GLC analysis was possible, SL-O-TT distributed rapidly. In these tissues, the maximum concentrations of SL-O-TT were measured 1 min after injection, with a drastic decline thereafter (Table 1). Control extracts from brain contained a peak in the region where the SL-O-TT peak appears. Extracts from kidney were so viscous in nature that the GLC column was rendered inoperable. For these reasons, no GLC data on brain were obtained, and only 1-min samples of kidney were analyzed. The GLC chromatograms for all the tissues analyzed did not show the metabolite peak at RT 7.1 in detectable concentrations as the plasma samples did. We therefore studied the peak corresponding to the parent compound SL-O-TT. In general, 5 min after injection either SL-O-TT was not detected, or tissues showed the lowest detectable concentrations. Thus, at 5 min, heart, lung, and skeletal muscle showed their lowest

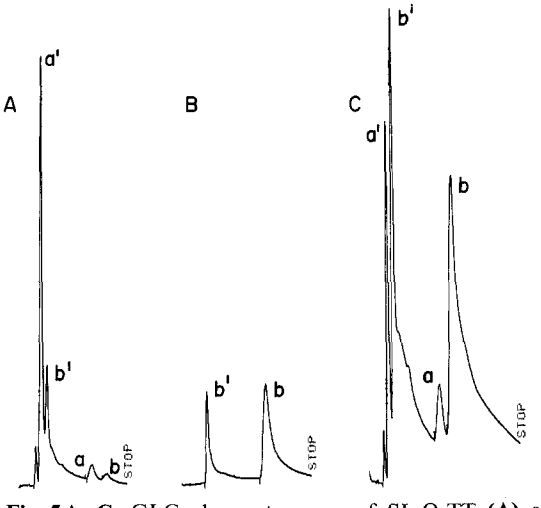

Fig. 5 A–C. GLC chromatograms of SL-O-TT **(A)** and SL-O-T **(B)**, and cochromatogram of both **(C)**. The GLC conditions were as in Fig. 2.

Table 1. Concentrations of SL-O-TT in plasma of mice after LV, injections of 10 mg/kg SL-O-TT

Time (min)	Plasma (mg/ml) GLC SL-O-TT	Metabolite	Total	Plasma (mg/ml) ESR Spin label
1	20.4 ± 1.02[a]	ND[b]	20.4	24.3 ± 8.4
3	8.95 ± 1.00	3.38 ± 0.73	12.3	12.9 ± 2.2
5	6.46 ± 0.58	5.13 ± 0.24	11.6	12.1 ± 2.4
10	3.79 ± 0.31	5.61 ± 0.53	9.40	11.9 ± 4.3
15	2.19 ± 0.24	4.07 ± 0.22	6.26	10.7 ± 4.4
30	0.95 ± 0.11	3.35 ± 0.41	4.30	9.27
60	0.41 ± 0.07	2.06 ± 0.40	2.47	ND
120	0.11 ± 0.01	0.90 ± 0.05	1.01	ND

[a] Mean ± SE of determination from three mice/time
[b] Not detectable

concentrations at 1.57 μg/g, 0.63 μg/g, and 1.25 μg/g, respectively (Table 2). Initially, except for heart and lung, tissue concentrations of SL-O-TT were lower than the concomitant plasma concentration. At 3 min after the injection the SL-O-TT concentrations in all tissue were lower than the concomitant plasma concentration.

Electron Spin Resonance Spectroscopy. The results of the ESR analysis are consistent with those of the GLC analysis in that the concentrations detected were highest at 1 min after injection and abruptly declined to low or undetectable levels. In skeletal muscle and lung tissues concentrations were detected by ESR but not by GLC 15 min after injection. The concentrations of spin label detected by ESR were consistently higher than those detected by GLC. The highest concentration detected was that in the lung at 143.7 μg/g, which quickly declined tenfold by 3 min and was not detected after 5 min. As mentioned before, ESR does not detect metabolites, but only spin label concentrations. Therefore, any alterations in the molecule other than the spin label will not be detected. The only metabolite that can change (diminish) the strength of the ESR signal is reduced SL-O-TT, because the hydroxylamine metabolite is diamagnetic. Thus, it is possible that the discrepancy in concentrations found between the two methods is a reflection of the metabolic activity of the tissues. Thus, liver, with a parent compound concentration of 0.59 μg/g according to GLC and a concentration of total label of 43.8 μg/g according to ESR shows the highest metabolic activity, while heart shows the lowest metabolic activity, with values of 54.3 μg/g SL-O-TT according to GLC and 88.8 μg/g label according to ESR (Table 2). Lung is an exception to the above observation, but a high SEM, on both GLC and ESR measurements makes these two values comparable. In lung, furthermore, 3 min after injection the SL-O-TT concentration according to GLC is nearly 100 times less than that measured by ESR.

In general, the tissue distribution measurements show more variability than plasma measurements in both GLC and ESR determinations. In some cases, one sample out of three gave ESR signals. It is possible that this is a reflection of a combination of drug solubility, tissue binding,

Table 2. Concentrations of SL-O-TT in tissues of mice after IV injections of 10 mg/kg SL-O-TT[a, b]

Time (min)	Brain[c] GLC	ESR	Heart GLC	ESR	Lung GLC	ESR	Liver GLC	ESR	Kidney[c] GLC	ESR	Spleen GLC	ESR	Skeletal muscle GLC	ESR
1	d	143.7[e] ± 73	54.3 ± 13	88.8 ± 10	76,8 ± 34	41.5 ± 10.9	0.59 ± 0.39	43.8 ± 8.6	11.33 ± 5.35	36.2 ± 7.12	1.92[e]	36.3	12.70	39.3 ± 7.94
3		10[f]	1.4 ± 0.25	ND	0.42 ± 0.03	45[f]	ND	ND	–	ND	ND	7.59 ± 1.13	2.66 ± 0.03	9.59 ± 2.77
5		ND[g]	1.57 ± 0.28	ND	0.63 ± 0.016	23.4 ± 10.4	ND	–	–	–	ND	5.83 ± 0.58	1.25 ± 0.31	9.38 ± 0.85
10		ND	ND	–	ND	–	–	–	–	–	–	7.85 ± 2.62	ND	6.01[e]
15		ND	ND	–	ND	33.4[f]	–	–	–	–	–	ND	–	5.6[e]

[a] μg/g
[b] Mean ± SEM of determination from organs from three mice/time
[c] Brain and kidney gave a viscous extraction which dirtied the GLC column
[d] Sample not analyzed
[e] Two samples out of three had a detectable signal
[f] One sample out of three had a detectable signal
[g] Not detectable

and metabolic reduction of the spin label to hydroxylamine. Of these, the first two possibilities can affect the drug's extraction from individual tissues. The last possibility simply decreases the amount of spin label detected. Concentrations of reduced SL-O-TT or SL-O-T were difficult to estimate because the internal standard required for quantitation of the drugs interfered with peaks a' or b'.

Discussion

Our results show that one of the main metabolites of spin-labeled thio-TEPA is spin-labeled TEPA. Oxidative desulfurization is known to occur in phosphorothioate (RO_2) P SX insecticides, which lead to powerful anticholinesterase metabolites [10]. It is possible that a similar mechanism is involved in SL-O-TT metabolism. Assuming that thio-TEPA and SL-O-TT are metabolized in a similar fashion, our result is in contrast with that of Craig et al. [1], who reported no TEPA resulting from the metabolism of thio-TEPA in mice. Our previous pharmacokinetic work with thio-TEPA also did not yield TEPA as a metabolite [3]. Further studies in our laboratory indicated that the ethyl acetate extraction procedure used for thio-TEPA was entirely unsuitable for the measurement of TEPA. A new extraction procedure based on sodium chloride and chloroform proved to be ideal for the extraction of TEPA. With this procedure, both TEPA and thio-TEPA exhibit well-resolved peaks on GLC, and TEPA was detected as a metabolite in mice treated IV with thio-TEPA [4]. A systematic search for other metabolites revealed reduced nitroxyl radicals, free spin label, and chlorinated compounds of the form $NHCH_2CH_2Cl$. Reduced nitroxyl radicals label did not show a GLC peak under our conditions. Free spin label should give a distinct ESR spectrum if it is tumbling faster than SL-O-TT. There was no evidence of a fast-rotating component in our ESR data. Chlorinated compounds yielded variable results on thin-layer chromatography. Despite our failure to identify free spin label and chlorinated compounds derived from opened aziridine rings, these metabolites and other phosphorus compounds cannot be ruled out at this time. Both GLC and ESR analysis indicate that there is very little nitroxyl radical reduction in plasma for up to 30 min after the bolus injection, if samples are kept frozen at $-20\,^{\circ}C$ and studied within 7–10 days after the experiment. Samples analyzed 4 weeks to 3 months after the experiments showed either a small ESR signal or none at all, while GLC chromatograms showed an increase in a' and b' peaks with a concomitant decrease in the a and b peaks. This result indicates a reduction of the label at slow rates even in frozen samples. Immediate analysis of plasma samples by ESR does yield reliable data. This result is important when considering that most reoxidation techniques require relatively large amounts of sample, some of which is inevitably lost.

Because we are interested in improving all aspects of the present antitumor agents, it is instructive to compare the pharmacokinetics of SL-O-TT with that of thio-TEPA. In both cases, plasma pharmacokinetics are biexponential, with slightly longer half-lives for SL-O-TT than for thio-TEPA. Thus thio-TEPA has a $t_{1/2}$ of 0.21 min for the distributive phase and a terminal $t_{1/2}$ of 9.6 min [3]. SL-O-TT, in contrast, has a distributive $t_{1/2}$ of 2.5 min and a terminal $t_{1/2}$ of 26.6 min. The apparent volume of distribution for SL-O-TT is larger (53.5 ml) than the weight of the animals (approx. 40 g). This result implies some sequestration of the drug not found in thio-TEPA. This result is not surprising, however, because of the higher lipophilicity of SL-O-TT. This characteristic of the drug is probably best seen in the tissue distributions where very large amounts are observed 1 min after injection. Unlike thio-TEPA, SL-O-TT shows abrupt declines in concentrations 3 min after injection. This behavior is also reflected in the ESR measurements for total label concentrations. For thio-TEPA, the decline of drug concentrations with time is more uniform. For instance, the concentrations of thio-TEPA in lung decrease from 1.5 μg/g at 1 min after injection to 1.1 μg/g 3 min after injection [3], whereas SL-O-TT concentrations decrease from 76.8 μg/g at 1 min after injection to 1.4 μg/g 3 min after injection (Table 2). This abrupt change is also reflected in the ESR data (Table 2). One possible explanation for this behavior is the difference in solubility of the drugs, but it could also very well be that 3 min after injection a great deal of the drug is bound and/or found in places where the extractants are ineffective. Comparison of ESR and GLC data allows the detection of metabolic activity in tissues. As mentioned earlier, liver shows the highest metabolic activity for SL-O-TT and heart the lowest. This result is also found with thio-TEPA. Thus, concentrations of thio-TEPA at 1 min after injection were lowest in liver (0.17 μg/g) and highest in heart (2.0 μg/g) [3].

Our results indicate that, as in radiolabeled drugs, a spin label can be used to detect changes in label concentrations that may or may not reflect changes in the molecule itself. The advantage of spin labeling over radiolabeling is that spin labels are not health hazards and are relatively easy to synthesize. The disadvantage is that the label is metabolically reduced to a diamagnetic compound not detectable by ESR. The use of two analytical methods is essential to avoid pitfalls that can be made when relying only on ESR. For intance, Emanuel et al [5] reported a high affinity of SL-NH-TT (Fig. 1) for tumors on the basis of spin label concentrations determined by ESR. While these results may be true, it remains to be seen whether or not the spin label in the tumor reflects SL-NH-TT levels, an active or inactive metabolite, or the label alone.

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Received January 14, 1985/Accepted February 28, 1985