ABSTRACT

Because the transport and accumulation of thiotapec by cells has not been characterized, these processes were investigated with $[^{14}C]$thiotepa and cultured L1210 or freshly obtained human or avian RBC. The octanol:phosphate buffered saline partition coefficient of thiotapec was 2.4 ± 0.1 ($n = 8$). With this value, the permeability coefficient ($P_s$) for thiotapec was estimated to be 2.8 × 10$^{-6}$ and 1.81 × 10$^{-3}$ cm/s and the half-life of accumulation of thiotapec by L1210 cells was estimated to be 0.063–0.40 s. Thiotapec accumulation by cells was measured after incubation of cells with $[^{14}C]$thiotepa and subsequent harvesting of cells by centrifugation through silicone fluid. Thiotapec accumulation by L1210 cells was biphasic. The initial phase was rapid and essentially complete by 10 s. The amount of cell-associated $^{14}$C increased linearly with increasing extracellular concentrations of thiotapec or with increasing size of the cell pellet. The absolute amount of cell-associated $^{14}$C was consistent with that expected if the $[^{14}C]$thiotepa had been evenly distributed in the incubation medium and a volume equal to that of the cell pellet had been sampled and counted. This rapid phase of thiotapec accumulation was not slowed when cells were incubated on ice. The second phase of $[^{14}C]$thiotepa accumulation occurred at a rate much slower than that of the initial phase. This slower phase of drug accumulation was linear for at least 5 h. The rate of $^{14}$C accumulation increased progressively over a range of extracellular thiotapec concentrations between 5 and 100 nmol/ml and could not be saturated under acceptable tissue culture conditions. The slower rate of $^{14}$C accumulation was ablated by incubating cells on ice and was reduced by 30–50% in the presence of 1 mM sodium azide or 2,4-dinitrophenol. The slow rate of accumulation of $^{14}$C reflected saturation of a relatively stable or constant amount of exchangeable $^{14}$C and an amount of nonexchangeable $^{14}$C which increased linearly from almost undetectable levels at the start of the experiment to amounts approximately equal to those of exchangeable radioactivity after 5 h. The initial association of $[^{14}C]$thiotepa with both human and avian RBCs was very rapid. Avian RBCs also exhibited a slow rate of $^{14}$C accumulation which was linear for at least 5 h but which was 15–20% that of L1210 cells. Human RBCs did not exhibit a slower rate of $^{14}$C accumulation and essentially all of the $^{14}$C associated with human RBCs was exchangeable for the 5 h duration of the experiment. All of the $^{14}$C associated with cells after 10 s or 5 h was accounted for by two radioactive spots on thin layer chromatography. One of these had an $R_f$ value identical to that of thiotepa and the other remained at the origin. There was no radioactivity in the area of the thin-layer chromatography plate corresponding to tepa. The radioactivity at the origin accounted for a greater proportion of the total radioactivity associated with L1210 cells incubated for 5 h with $[^{14}C]$thiotepa than it did in L1210 cells incubated with radiolabelled drug for a few seconds.

INTRODUCTION

Thiotepa2 is an antitumor agent that has been used clinically for over 30 years (1). Thiotapec belongs to a family of alkylating agents that contain pentavalent phosphorus and aziridine moieties. In current medical practice, thiotapec is administered systematically to treat metastatic carcinoma of the breast (2, 3), intravesically to treat superficial carcinoma of the bladder (4, 5), and intrathecally to treat carcinomatous meningitis (6, 7). Recently there has been a resurgence of interest in thiotapec primarily because of its use at high doses in conjunction with autologous bone marrow transplantation (8–14). Pharmacological data on thiotapec is incomplete, perhaps reflecting development of the drug at a time when tissue culture and analytical chemical techniques were still relatively crude. With the development by Grochow et al. of a specific and facile gas chromatographic analysis of thiotapec (15) and the subsequent modification and variation of this method (14, 16–19) to allow measurement of tepa, there has been a concerted effort by a number of laboratories to define the pharmacokinetics of thiotapec when given by standard means as well as at the high doses used with autologous bone marrow transplantation (14, 20–25). The cellular and biochemical aspects of thiotapec pharmacology have been less well characterized. Recent investigations have explored the in vitro interaction of thiotapec and tepa with DNA and the effects of these compounds on cellular growth and macromolecular biosynthesis in vitro (26–31). Although in vivo pharmacokinetic studies indicate rapid distribution of thiotapec to tissues (16), the means by which thiotapec enters and is accumulated by cells is an area as yet unexplored. The studies presented in this paper were undertaken specifically to address this lack of information and with the hope that the development of data with regard to cellular transport and accumulation of thiotapec might translate into more optimal clinical use of the drug.

MATERIALS AND METHODS

Drugs and Drug Purity. [$U^{-14}$C]Thiotepa (2.3 mCi/mmol) was produced by reaction with chloro-$[U^{-14}$C]ethylamine hydrochloride with sodium hydroxide, followed by reaction of the resulting [$U^{-14}$C]aziridine with thiophosphoryl chloride. The resulting radiolabelled thiotapec was then purified by column chromatography followed by crystallization. This material was custom synthesized by Amersham Corporation (Arlington Heights, IL). Nonradioactive thiotapec and tepa were graciously supplied by Lederle Laboratories (Pearl River, NY) and Dr. Peter L. Gutierrez, respectively.

Radiochemical purity of the final [$U^{-14}$C]thiotepa preparation was determined by thin layer chromatography (TLC) on 250-μm silica gel 60 plates (E. Merck, Darmstadt, FRG) that were developed to 15 cm in an ascending fashion with either 100% ethyl acetate or chloroform:acetone (4:1, v/v). Nonradioactive thiotapec and tepa standards were included on each plate. Thiotapec and tepa were visualized by reaction with NBP (Sigma Chemical Co., St. Louis, MO) (22). To accomplish this, plates were sprayed sequentially with 0.2 M sodium acetate buffer, pH 4.6, and a 5-g/100-ml solution of NBP in acetone, heated at 100°C for 30 min, and finally, sprayed with 1 N sodium hydroxide. The blue NBP-reactive spot corresponding to thiotapec and 1-cm segments of the remainder of the lane were scraped from the plate, placed into scintillation vials, and mixed with 0.1 ml glacial acetic acid and 5 ml Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA). Radioactivity was determined by a Beckman LS5801 liquid scintillation counter. Under these conditions, thiotapec had $R_f$ values of page charges. This article must therefore be hereby marked advertisement in lieu of page charges. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in lieu of page charges.
values of 0.30 and 0.44 in 100% ethyl acetate and chloroform:acetone (4:1, v/v), respectively, and radiochemical purity was greater than 98% in each system.

Reagents. SF 1250 silicone fluid was generously donated by the General Electric Company, Silicone Products Division (Waterford, NY). 2,4-Dinitrophenol and sodium azide were obtained from Sigma Chemical Co. and Fisher Scientific (Fair Lawn, NJ), respectively. The cell suspension was then transferred to a microcentrifuge tube containing 0.4 ml SF 1250 silicone fluid and was centrifuged and assayed for cellular radioactivity as described above.


determinations of cellular accumulation of thiotepa were performed and were supplied as a suspension produced by mixing freshly obtained cockerel blood with an equal volume of Alsever’s solution.

ams the overall tightness of the membrane, is defined as the diffusion constant for diffusion within the membrane divided by the operative thickness of the biological membrane, and $S_m$ is the differential mass selectivity coefficient.

The half-life ($t_{1/2}$) of entry of thiotepa into L1210 cells was estimated by integration of Pick’s law of diffusion (37), i.e.:

$$t_{1/2} = \frac{V}{S_m} \ln 2 \tag{B}$$

where $V$ is the cell volume and $A$ is the cell surface area.

Incubation of Thiotepa with Cells. L1210 cells were washed twice with PBS, resuspended in fresh medium A, and added to an equal volume of medium A that contained the desired concentration of radiolabeled thiotepa. Freshly obtained heparinized human whole blood and avian RBC suspensions were diluted 1:20 and 1:10, respectively, in medium A and the resulting RBC preparations were mixed with equal volumes of medium A that contained the desired concentration of radiolabeled thiotepa. The majority of incubations were performed at 37°C in an atmosphere of 5% CO$_2$, 95% air, and 95% humidity. Experiments with incubation times up to 3 min used a final incubation volume of 1 ml and were performed in 13- x 100-mm glass tubes. Experiments with longer incubation times used a final incubation volume of 5 ml and were performed in 25-cm$^2$ tissue culture flasks.

These experimental conditions were based on control experiments which documented markedly reduced viability of cell cultures maintained for more than an hour at cell concentrations of 2 x 10$^7$/ml or 10$^7$/ml in 16- x 150-mm glass tubes that were held at a 15° angle from the horizontal. Control experiments also showed no difference in thiotepa accumulation by L1210 cells in medium A or in a solution of PBS containing 0.1 mM bovine serum albumin and 0.25 g/l glucose (38). In some experiments, 1 mM sodium azide or 2,4-dinitrophenol were included in the incubation medium, and in certain others, incubations were performed on ice in the 5% CO$_2$, 95% humidity atmosphere described earlier. All experiments were performed in triplicate and were repeated at least once.

Assay of Cellular Drug Accumulation. At the conclusion of the specified incubation time, cells were harvested by centrifugation through SF 1250 silicone fluid (39). For experiments with incubation times less than 3 min, the entire 1-ml incubation volume was transferred to a 1.5-ml microcentrifuge tube containing 0.4 ml of SF 1250 silicone fluid, and the tube was centrifuged at 13,000 × g for 30 s in a Beckman Microfuge E. Medium containing the drug and the underlying silicone fluid layer were removed by aspiration, and the walls of the tubes were wiped carefully. Sedimented cells were transferred to scintillation vials with two 0.5-ml rinses of distilled water. 5 ml of Ready Safe scintillation fluid were added to the vials, and radioactivity was determined with a Beckman LS5801 liquid scintillation counter. For experiments with longer incubation times, each 5-ml incubation volume was transferred to a 16- x 150-mm glass tube, centrifuged at 1150 × g for 10 min, and 4 ml of the resulting supernatant medium were removed. Sedimented cells were resuspended in the remaining 1 ml of medium. This 1-ml cell suspension was transferred to a microcentrifuge tube containing SF 1250 silicone fluid and was centrifuged and assayed for cellular radioactivity as described above. In control experiments to assess non-specific sedimentation of radioactivity by this centrifugation procedure, [14C]thiotepa was replaced by an equal amount of [14C]inulin, a molecule known to be restricted to the extracellular fluid and not to enter cells. Rates of accumulation of [14C]thiotepa were calculated by linear regression analysis of cellular [14C] (the dependent variable) versus time of incubation (the independent variable). These rates and the extracellular concentrations of thiotepa associated with them were used in attempts to calculate Michaelis-Menten kinetic characteristics, such as $K_m$ and $V_{max}$, for the process of [14C]thiotepa accumulation by L1210 cells.

Efflux Experiments. After incubation with 50 nmol/ml [14C]thiotepa for 0, 3, or 5 h, L1210 cells or RBC were transferred to 16- x 150-mm glass tubes and sedimented by centrifugation at 1150 × g for 10 min. The supernatant fluid, containing the radiolabeled drug, was removed and the sedimented cells were resuspended in 10 ml of drug-free medium A. After incubation for an additional 15 min at 37°C in an atmosphere of 5% CO$_2$, 95% air, and 95% humidity, cells were again sedimented by centrifugation at 1150 × g for 10 min. Nine ml of the resulting supernatant medium were removed, and the sedimented cells were resuspended in the remaining 1 ml of medium. This 1 ml of cell suspension was then transferred to a microcentrifuge tube containing 0.4 ml SF 1250 silicone fluid and assayed for cellular radioactivity as described above.

Chromatographic Analysis of Thiotepa. In initial experiments, the stability of nonradiolabeled thiotepa incubated with cell concentrations up to 10$^7$/ml was investigated by gas chromatography. Cell suspensions were incubated with 5 nmol/ml thiotepa in medium A for up to 6 h at 37°C in an atmosphere of 5% CO$_2$, 95% air, and 95% humidity. At hourly intervals, aliquots were removed, cells were sedimented by centrifugation at 13,000 × g for 1 min, and 0.2-ml portions of the resulting supernatant medium were analyzed for thiotepa with the gas chromatographic method of Grochow et al. (15, 16).

The nature of cellular [14C] after incubation with [14C]thiotepa was investigated with TLC. Cells were incubated for 5 h with radiolabeled thiotepa as described earlier. After 5 h, cells were centrifuged through SF 1250 silicone fluid, the supernatant medium and SF 1250 silicone fluid were removed as described earlier, and the resulting cell pellet was mixed with 1 ml of absolute ethanol. Concomitant control cultures were incubated without thiotepa and handled as just described or had 1.5 × 10$^7$ DPM of [14C]thiotepa added to the cell pellet immediately
before the cells were denatured with ethanol. After being stored overnight at −20°C, the ethanol denatured cell suspensions were centrifuged at 13,000 × g for 1 min. The resulting supernatant fluids were transferred to clean microcentrifuge tubes and were evaporated to dryness under a stream of nitrogen. The dried residues were resuspended in small volumes of absolute ethanol and were spotted onto 250-μm silica gel 60 TLC plates that were subsequently developed in chloroform: acetone (4:1, v/v), sprayed with NBP, and counted for radioactivity as described earlier. Nonradioactive standards of thiotepa and tepa were included on each TLC plate.

RESULTS

Permeability Coefficient and Half-Life of Accumulation of Thiotepa by L1210 Cells. Thiotepa proved lipophilic with an octanol:PBS partition coefficient of 2.4 ± 0.1 (n = 8). With this value and the relationship, $P_s = P_a M^d K^b$ (36), it was possible to calculate the approximate $P_s$ for thiotepa and subsequently to estimate the $t_0$, of accumulation of thiotepa by L1210 cells (37). In calculating $P_s$, $S_a$ was assigned a value of 1 as suggested by Wolosin and Ginsburg (40), and the fact that the values of $P_s$ and $S_a$ are not known for L1210 cells led us to utilize an approach similar to that taken by Begleiter et al. in similar studies (41, 42). In this approach, a range of $P_s$ and $S_a$ values that have been established for a variety of cell types were entered into the equation to generate a realistic range of values for $P_s$ (Table 1). In this fashion, $P_s$ values of 2.82 × 10⁻⁴ to 1.81 × 10⁻³ cm/s were calculated for thiotepa (Table 1). With these $P_s$ values and the values of 476 μm² and 290 μm² for the volume and surface area, respectively, for L1210 cells, the $t_0$ of accumulation of thiotepa by L1210 cells could be predicted, as described in “Materials and Methods,” to be between 0.063 and 0.40 s.

Accumulation of [¹⁴C]Thiotepa by L1210 Cells. Cellular accumulation of [¹⁴C]thiotepa was a biphasic process. As predicted from the estimated $t_0$, of accumulation, the initial association of thiotepa with cells was extremely rapid (Fig. 1). By 6–10 s, which was the fastest that cells could be assayed, the rapid phase of cellular [¹⁴C] accumulation was essentially complete (Fig. 1). There was no significant increase in cell-associated [¹⁴C] between 10 and 180 s. In contrast, there was no cell-associated [¹⁴C] above background in control experiments in which [¹⁴C]-inulin was used in place of radiolabeled thiotepa.

Additional experiments were also consistent with the very rapid diffusion of thiotepa into cells. In the presence of a constant cell number, and therefore a constant cell pellet volume, there was a linear relationship between the extracellular concentration of [¹⁴C]thiotepa and the amount of [¹⁴C] associated with the cell pellet after 10 s of incubation (Fig. 2). Moreover, the absolute amount of [¹⁴C] measured in each cell pellet was consistent with what would be present if the [¹⁴C]thiotepa had been uniformly distributed throughout the incubation mixture and a volume equal to that of the cell pellet had been sampled and counted. Expressed another way, the percentage of radioactivity associated with the cell pellet remained essentially constant over a 250-fold range of thiotepa concentrations, and the percentage of total radioactivity associated with the pellet was consistent with the percentage of the total incubation volume accounted for by the cell pellet. Similarly, variation of the cell concentration, and thereby the size of the cell pellet, in the presence of a constant extracellular concentration of [¹⁴C]thiotepa produced a linear increase in the amount of [¹⁴C] associated with the cell pellet (Fig. 3). Again, the absolute amount of [¹⁴C] associated with each cell pellet was consistent with what would be predicted if the [¹⁴C]thiotepa had been uniformly distributed throughout the incubation medium and a volume equal to that of each cell pellet had been sampled and counted.

This rapid phase of drug accumulation was not demonstrably reduced when [¹⁴C]thiotepa accumulation was studied in cells incubated at 4°C.

Although the rapid phase of [¹⁴C]thiotepa accumulation was essentially completed by 10 s, a second, and much slower, phase of drug accumulation also occurred. This slower process of [¹⁴C] accumulation was linear for at least 5 h (Fig. 4), and the rate

<table>
<thead>
<tr>
<th>Source of Pₐ and Sₐ values (35)</th>
<th>Pₐ (cm/s)</th>
<th>Sₐ</th>
<th>Predicted Pₐ (cm/s)²</th>
<th>Predicted tₒ (s)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chara</td>
<td>0.13</td>
<td>2.9</td>
<td>1.81 × 10⁻³</td>
<td>0.063</td>
</tr>
<tr>
<td>Nitella</td>
<td>0.50</td>
<td>3.7</td>
<td>1.68 × 10⁻³</td>
<td>0.068</td>
</tr>
<tr>
<td>Beef RBC</td>
<td>5.0</td>
<td>6.0</td>
<td>2.82 × 10⁻¹</td>
<td>0.40</td>
</tr>
<tr>
<td>Arbacia egg</td>
<td>0.40</td>
<td>4.2</td>
<td>5.52 × 10⁻⁴</td>
<td>0.21</td>
</tr>
</tbody>
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* $P_s = P_a M^d K^b$.

* $t_0 = (V/2P)t_m$ where $V$ for L1210 cells = 476 μm³ and $A = 290 μm²$.

Fig. 1. Rapid phase of accumulation of [¹⁴C]thiotepa by L1210 cells. 10⁷ L1210 cells were incubated at 37°C with 5 nmol/ml [¹⁴C]thiotepa, and at the indicated times, cells were harvested by centrifugation through silicone fluid and cell-associated radioactivity was assessed. Points, means ± SD of two experiments, each performed in triplicate.

Fig. 2. Relationship between extracellular concentration of [¹⁴C]thiotepa and the rapid accumulation of drug by L1210 cells. 10⁷ L1210 cells were incubated at 37°C with the indicated extracellular concentrations of [¹⁴C]thiotepa. After 10 s, cells were harvested by centrifugation through silicone fluid and cell-associated [¹⁴C] was assessed. Points, means ± SD of four experiments, each performed in triplicate.

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CELLULAR ACCUMULATION OF THIOTEPA

Fig. 3. Relationship between cell pellet size and cell associated 14C after rapid phase of cellular accumulation of [14C]thiotepa by L1210 cells. Various numbers of L1210 cells were incubated in 1 ml at 37°C with 5 nmol/ml [14C]thiotepa. After 10 s, cells were harvested by centrifugation through silicone fluid, and cell-associated 14C was assessed. Points, means ± SD of two experiments, each performed in triplicate.

Fig. 4. Accumulation of 14C by L1210 cells incubated with [14C]thiotepa. 10^7 L1210 cells were incubated at 37°C with 5 nmol/ml [14C]thiotepa. At specified times, cells were harvested by centrifugation through silicone fluid, and cell-associated 14C was assessed. Points, means ± SD of a representative triplicate experiment.

The final experiments characterizing the accumulation of radiolabeled thiotepa by L1210 cells examined the exchangeability of cellular 14C after various periods of incubation (Fig. 7). After 10 s of incubation, L1210 cells incubated with 50 nmol/
**CELLULAR ACCUMULATION OF THIOTEPA**

![Graph](image)

**Fig. 6.** a, the effect of temperature on the slow rate of accumulation of ^14^C by L1210 cells incubated with [^14^C]thiotepa. 10^7^ L1210 cells were incubated at 37°C or on ice (O) with 50 nmol/ml [^14^C]thiotepa. After 0, 3, or 5 h, cells were harvested by centrifugation through silicone fluid, and cell-associated ^14^C was assessed. Points, means ± SD of a representative triplicate experiment. b, the effect of sodium azide or 2,4-dinitrophenol on the slow rate of accumulation of ^14^C by L1210 cells incubated with [^14^C]thiotepa. 10^7^ L1210 cells were incubated at 37°C with 50 nmol/ml [^14^C]thiotepa and 1 mM sodium azide (O), 1 mM 2,4-dinitrophenol (A) or no metabolic poison (O). After 0, 3, or 5 h, cells were harvested by centrifugation through silicone fluid, and cell-associated ^14^C was assessed. Points, means ± SD of a representative triplicate experiment.

ml [^14^C]thiotepa had accumulated an amount of ^14^C consistent with experiments described earlier, but after being resuspended and incubated for 15 min in drug-free medium, essentially all of the radioactivity was lost from the cells (Fig. 7). As expected, L1210 cells incubated with [^14^C]thiotepa for 3 or 5 h accumulated ^14^C linearly and at a rate consistent with earlier experiments. This increase, however, reflected the summation of a relatively constant amount of exchangeable ^14^C and an amount of nonexchangeable ^14^C which increased linearly from almost undetectable levels at the start of the experiment to amounts approximately equal to those of exchangeable radioactivity after 5 h of incubation (Fig. 7).

Accumulation of [^14^C]Thiotepa by Red Blood Cells. As with L1210 cells, the initial association of [^14^C]thiotepa with human and avian RBCs was very rapid and was essentially complete by 10 s of incubation. Although cockerel RBCs exhibited a second slower phase of ^14^C accumulation which was linear for at least 5 h, it was five to six times slower than that of L1210 cells. In contrast, human RBCs differed from L1210 cells and avian RBCs in that they did not exhibit a detectable slower rate of ^14^C accumulation. Moreover, essentially all of the ^14^C associated with human RBCs was exchangeable for the duration of the experiment.

**DISCUSSION**

The studies described in this communication fill a gap in the existing knowledge concerning the pharmacology of an alkylating agent whose clinical utility was established more than 30 years ago (1) and which is currently undergoing a reevaluation in clinical usage beyond those for which it has gained general acceptance (2–7). In addition, these studies, which represent an extension of our ongoing research program of thiotepa pharmacology, add to a body of literature of similar studies performed with other alkylating agents to which our results can be compared and contrasted (41–47).

Chromatographic Analysis of ^14^C Accumulated by L1210 Cells. In cells incubated with [^14^C]thiotepa for 10 s or 5 h, all of the ^14^C was accounted for by two radioactive spots on the TLC plate. One of these, with Rf = 0.44, corresponded to the authentic thiotepa standard, whereas the other represented ^14^C that remained at the origin. There was no ^14^C that migrated with an Rf greater than that of thiotepa, and there was no radioactivity with an Rf greater than 0 but less than that of thiotepa. Specifically, there was no ^14^C at the location corresponding to the authentic tepa standard. There was, however, a major difference in the nature of the ^14^C from cells that had incubated for 5 h with [^14^C]thiotepa as compared to that from cells to which [^14^C]thiotepa was added immediately before the cells were denatured with ethanol. In the latter cells, approximately 90% of the cellular ^14^C migrated with an Rf equal to that of thiotepa, with only 8–10% remaining at the origin. In contrast, only 50–70% of the ^14^C from cells that had incubated with [^14^C]thiotepa for 5 h migrated as did thiotepa, with the other 30–50% remaining at the origin. This ^14^C, which did not migrate up to the TLC plate in chloroform:acetone (4:1, v/v), could be quantitatively extracted from the silica gel with absolute methanol and when rechromatographed again remained at the origin with none migrating as thiotepa or tepa.

The biphasic accumulation of thiotepa by L1210 cells almost certainly reflects two different processes. The initial rapid phase...
of thiotepa accumulation is compatible with simple diffusion. The rapidity of thiotepa's initial association is quantitatively consistent with the octanol:PBS partition coefficient measured and the range of permeability coefficients and half-lives of accumulation predicted based on that partition coefficient. More specifically, the shortest time after thiotepa addition that cells could be assayed still exceeded the five half-lives of accumulation that would be required to achieve steady state. Furthermore, the degree of cellular drug accumulation with respect to the overall incubation volume, the lack of effect of temperature and metabolic poisons, and the inability to saturate the rapid phase of thiotepa accumulation by L1210 cells are all consistent with a process of simple diffusion (35). The rapidity of the initial phase of thiotepa accumulation is also consistent with the results of in vivo pharmacokinetic studies which show very rapid and wide tissue distribution of thiotepa after i.v. injection and which have indicated the volume of distribution of thiotepa to be approximately equal to total body water (16). On the other hand, our studies do not localize the cell-associated 14C as being intracellular, and the possibility that some or even all of the 14C rapidly associated with cells is bound to or held in the cell membrane cannot be ruled out.

The second, and slower, phase of thiotepa accumulation by L1210 cells is likely to reflect alkylation of cellular constituents and/or metabolism of the drug to more polar forms. It is evident that the second, slow phase of thiotepa accumulation is primarily due to accrual of nonexchangeable 14C, a process compatible with accumulation of bound radiolabel or, less likely, a very polar metabolite. The ablation of the second phase of thiotepa accumulation by reduced temperatures and its slowing by metabolic poisons imply that it is a function associated with viable cells which have metabolic capability. That the slowing of thiotepa accumulation after the brief initial rapid phase reflects cell death is argued against by our parallel control studies which documented viable cells. However, it is once again impossible to rule out the less likely possibility that the second phase of thiotepa accumulation reflects slow internalization of radiolabel that was initially rapidly bound to cell membranes.

Our studies with avian and human RBCs provide some further insight into the accumulation of thiotepa by L1210 cells. The fact that RBC-associated 14C is at steady state by 6–10 s is again compatible with free diffusion of thiotepa into cells: however, again membrane binding cannot be ruled out. The fact that human RBCs do not exhibit a second, slow phase of thiotepa accumulation and do not acquire 14C in a nonexchangeable form, as do L1210 cells, may reflect obvious and important differences in cell structure between the two cell types. Unlike L1210 cells, human RBCs have no nuclei and are virtually devoid of mitochondria and RNA. They thereby lack nucleic acids for thiotepa to alkylate and have fewer types of enzymes that might convert thiotepa into a nonexchangeable form. The fact that avian RBCs, which are nucleated, exhibit a slow phase of 14C accumulation when incubated with [14C]thiotepa lends further weight to this argument.

An obvious extension of the current transport studies is to define more precisely the nature of nonexchangeable 14C in L1210 cells. The TLC studies reported herein, while not defining the structure of the nonexchangeable 14C, do provide important data in that no tepa was observed in any cell extract. This apparent inability of L1210 cells to convert thiotepa to tepa is consistent with in vivo studies which implicate the liver as the major source of this metabolic process (16). The radiolabeled material that remained at the origin, and that increased with increasing time of incubation of thiotepa with cells, could represent 1 or more chemical moieties. It might be thiotepa with 1 or 2 aziridines removed to produce a compound which would interact tightly with the TLC plate silica gel coating. It might represent thiotepa with 1 or more aziridines converted to β-hydroxyethyl moieties which would also be very polar. Alternatively, it might represent thiotepa or a radiolabeled aminooethyl residue bound to small peptides or low molecular weight nucleic acids that were not precipitated in iced ethanol and which did not migrate in the TLC system used in these studies. Ongoing in vitro studies in our laboratory have begun to investigate the interaction of thiotepa with DNA in cell-free systems (48) and will be extended to pursue this issue in cells.

Finally, consideration of the current studies in light of other aspects of thiotepa's pharmacokinetic behavior may provide further support for tepa or another metabolite of thiotepa accounting for an important part of thiotepa's cytotoxic activity. These pharmacokinetic characteristics include the relatively short plasma half-life of thiotepa (14–16, 19–25) and the much longer plasma half-life of tepa (19–23) as well as the fact that other alkylating moieties are excreted in the urine of patients treated with thiotepa long after the urinary excretion of thiotepa and tepa has ceased (20). Still, not all of thiotepa's cytotoxic activity can be attributed to tepa or another metabolite. Thiotepa is cytotoxic in vitro in systems where there is no evidence of conversion of thiotepa to tepa, unless the rate of that conversion is very slow in comparison to the reaction of tepa with nucleic acids or its conversion to other cytotoxic metabolites that are not measured by current techniques. Thus, despite the recent great increase in pharmacokinetic and pharmacological knowledge concerning thiotepa, much remains to be learned and ideally translated into more optimal clinical use of this agent.

ACKNOWLEDGMENTS

We thank B. Knickman for excellent secretarial assistance in preparation of this manuscript and Lederle Laboratories for partial support of this project.

REFERENCES


Cellular Transport and Accumulation of Thiotepa in Murine, Human, and Avian Cells

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