In Vivo Pharmacokinetics of Triazinate in L-1210 and W-256 Cells

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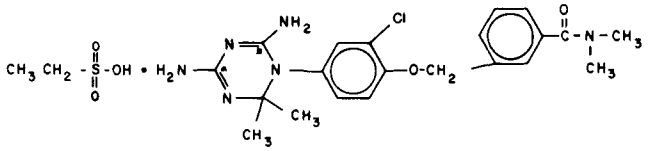
Abstract □ A pharmacokinetic model for triazinate uptake in L-1210 cells in mice and W-256 cells in rats was developed to describe the observed concentration profiles with time in these cells following a 36 mg/m^2 ip injection. The L-1210 cell permeability to triazinate was found to be ~15 times smaller compared with W-256 cells. Similarly, the partition coefficient for L-1210 cells was calculated to be ~175 times smaller than for W-256 cells. Cell membrane permeability appears to be the key parameter determining drug transport at a short time after injection.

Keyphrases □ Pharmacokinetics—of triazinate *in vivo* in L-1210 and W-256 cells □ Binding—*in vivo* pharmacokinetics of triazinate in L-1210 and W-256 cells □ Permeability, cell membrane—*in vivo* pharmacokinetics of triazinate in L-1210 and W-256 cells

Triazinate[1] (I) (NSC-139105) is a triazine folate antagonist which inhibits the enzyme dihydrofolate reductase in nucleic acid synthesis. Triazinate penetrates cells passively (1) unlike another prominent dihydrofolate reductase inhibitor, methotrexate, which enters cells by active transport. Although effective in the cure of Walker 256 ascites tumors, triazinate is not active against the L-1210 leukemia (2). This contrast in antineoplastic activity appears related to the significant accumulation of triazinate in the W-256 cells compared with the L-1210 cells (3). Since differences in drug transport probably account for the marked differences in triazinate activity observed in these cell lines (3), a quantitative analysis of triazinate transport and uptake through a mathematical model may be useful in understanding this behavior.



I

A preliminary pharmacokinetic model for triazinate transport in L-1210 leukemia and W-256 ascites cells is reported based on the uptake data in tumor-bearing mice and rats of Cashmore *et al.* (2). The model parameters include: cell permeabilities, drug–dihydrofolate reductase binding constants, and drug partition coefficients.

EXPERIMENTAL

Details of the experimental procedures are given elsewhere (2). Normal male rats weighing 55 to 60 g were given intraperitoneal inoculations of 5 × 10^6 W-256 ascites cells; normal male mice weighing 25 to 30 g were given intraperitoneal inoculations of 5 × 10^6 L-1210 ascites cells. Four days later, the animals were given intraperitoneal injections of 0.2 μCi[^{14}C]triazinate (0.3 μCi/μmole); this corresponded to dosage levels of 36 mg/m^2 (12 mg/kg in mice and 6 mg/kg in rats).

At intervals from 15 min to 6 hr, three animals of each species were sacrificed. Twelve milliliters of Eagle's minimal essential medium were then injected into the peritoneal cavity, and the ascites cells and fluid were removed. After separation by centrifugation, the cells and supernatant fluids were stored frozen.

The ascites cells were lysed by freeze-thawing twice in four volumes of 0.9% NaCl solution. After centrifugation, 0.5 ml of each supernatant was added to 10.0 ml of scintillation fluid[2] and counted. The counting efficiency, as determined by external standardization and standard curves for the carbon 14 radioactivity in the scintillation fluid, was 80–85%.

Thin-layer chromatography was used to determine whether triazinate was metabolized when administered *in vivo*. Silica gel glass plates[3], 5 × 20 cm, with 0.25-mm coating, were prewashed with ethyl acetate. The solvent system of either 2-methoxyethanol–ethanesulfonic acid (1000:3) or 2-methoxyethanol–glacial acetic acid (1000:3) was used. One milliliter

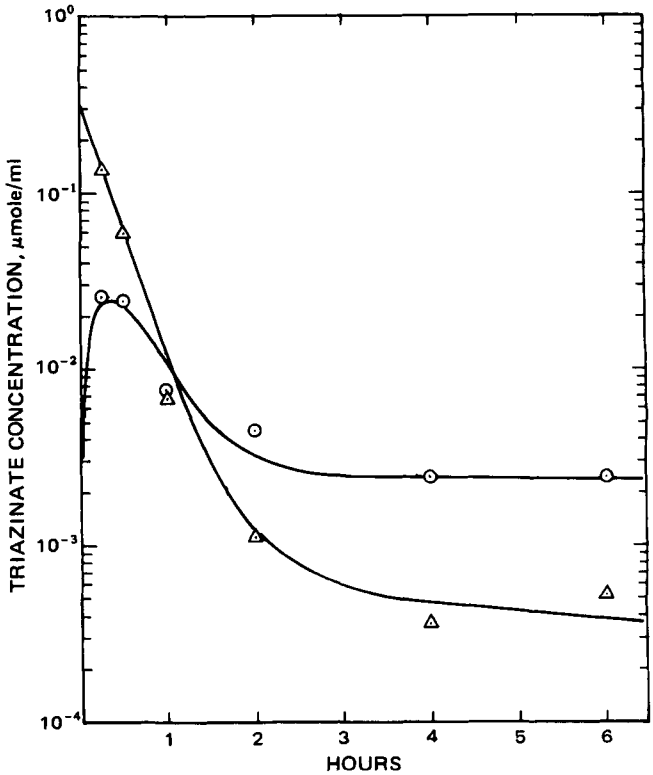


Figure 1—*Concentrations of triazinate in the peritoneal fluid and L-1210 cells as a function of time following injections of 12 mg/kg ip (36 mg/m^2) in mice. Points represent experimental determinations (2). The solid lines represent the peritoneal fluid-forcing function (C_{pe}) and the model predictions of L-1210 cell triazinate concentrations (C_c). The peritoneal fluid triazinate concentration was fitted by the expression:*

$$C_{pe} = 0.286\, e^{-0.0691t} + 0.0741\, e^{-0.0414t} + 0.000707\, e^{-0.00165t}$$

Other parameters are given in Table I. Key: (⊙) L-1210 cells; (△) peritoneal fluid.

[1] Triazinate: ethanesulfonic acid compounded with α-(2-chloro-4-(4,6-diamino-2,2-dimethyl-*s*-triazine-1(2*H*)-yl)phenoxy)-*N*,*N*-dimethyl-*m*-toluamide (1:1).

[2] Aquafluor.

[3] Brinkmann Silica Gel F-254.

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Table I—Model Parameters for L-1210 Ascites Cells in Male Mice and W-256 Ascites Cells in Male Rats

Parameter	Mice (L-1210 cells)	Rats (W-256 cells)
V_c, cell volume, ml	0.21[a]	
V_{pe}, peritoneal cavity volume, ml	1.5[b]	3.1[b]
a, dihydrofolate reductase binding constant, μmole/ml	2.3×10^{-3} [c]	2.2×10^{-4} [d]
ϵ, dissociation constant, μmole/ml	2.2×10^{-8} [e]	
Average protein content[f]		
Cells, mg	69	140
Peritoneal fluid, mg	32.4	56.1
Estimated physicochemical parameters		
PA/V_c, cell permeability or passive transfer coefficient, min^{-1}	0.01	0.15
R, fluid–cell partition coefficient, dimensionless	0.2	35

[a] Calculated from average of experimental measurements of L-1210 cell numbers with time (2) and properties of tumor cells given by Sirotnak and Donsbach(4). [b] Estimated from values of Weissbrod *et al.* (5). [c] Adapted from Goldman *et al.* (6). [d] From Werkheiser (7). [e] Methotrexate dissociation constant from Bischoff *et al.* (8). [f] Experimentally measured.

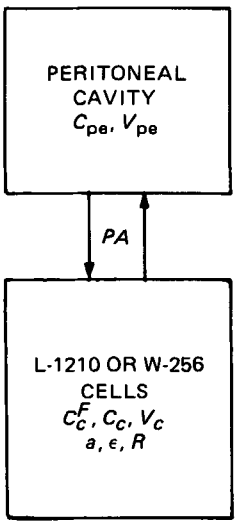
of methanol was added to 0.5 ml of crude cell extract, mixed, and centrifuged, and the resulting solution was evaporated to 0.2 ml. Five microliters of this solution was applied onto the silica gel plates. Reference and control samples consisting of [^{14}C]triazinate, unlabeled triazinate, and control tissue extracts plus [^{14}C]triazinate were run with each set. The plates were run for 3 hr at room temperature and then air dried. Examination under UV light revealed a fluorescent green background with dark quenching spots. The spots were scraped and placed in a counting vial containing the scintillation fluid, and the radioactivity was determined.

No evidence for drug metabolism was obtained using the methanol extracts of tumor cells, as well as liver, kidney, and spleen cells, obtained up to 8 hr after drug administration. Analysis of methanol extracts of urine and feces also indicated no evidence for metabolism of ^{14}C-labeled triazinate in mice or rats.

The values of drug concentrations (in micromoles per milligram of protein) reported previously (2) were converted into micromoles per gram of cells or micromoles per milliliter of ascites fluid in this study using the values given in Table I.

MODEL DEVELOPMENT

A schematic of the model for triazinate pharmacokinetics in tumor cells is shown in Scheme I. The drug is injected into the peritoneal cavity, from which it enters the tumor cells and other body tissues and blood. Previous investigators of methotrexate transport *in vivo* simulated plasma or peritoneal fluid drug concentrations with multiple exponential expressions, which then served as inputs to the tumor model simulations (5, 9, 10). This approach focuses on drug kinetics in the tumor cells and obviates the need to consider details of triazinate distribution in the rest of the body.



Scheme I—*The pharmacokinetic model for triazinate in L-1210 and W-256 cells. Symbols are defined in the text.*

In this study, instantaneous mixing of drug in the peritoneal cavity following intraperitoneal injection is assumed. A three-term exponential decay function of the form:

$$C_{pe} = a_1 e^{-b_1 t} + a_2 e^{-b_2 t} + a_3 e^{-b_3 t} \quad \text{(Eq. 1)}$$

is fit to the available peritoneal fluid concentration (C_{pe}) data as a function of time (t). (Here, $a_1 - a_3$ and $b_1 - b_3$ are empirical constants.) This expression serves as a forcing function in the mass balance for drug accumulation in the tumor cells:

$$\frac{dC_c}{dt} = \left(\frac{PA}{V_c}\right)\left(C_{pe} - \frac{C_c^F}{R}\right) \quad \text{(Eq. 2)}$$

where V_c is the volume of the tumor cells in milliliters; C_c is the total concentration of free and bound triazinate in the cells in micromoles per milliliter; PA is the cell permeability to drug in milliliters per minute; C_{pe} is the triazinate concentration in the peritoneal fluid in micromoles per milliliter; C_c^F is the concentration of free triazinate in the cells in micromoles per milliliter; and R is the partition coefficient relating peritoneal fluid concentration to free tumor cell concentration of triazinate at equilibrium (dimensionless). Drug transport in the model is considered to occur by passive diffusion, with drug binding to dihydrofolate reductase occurring in the cells.

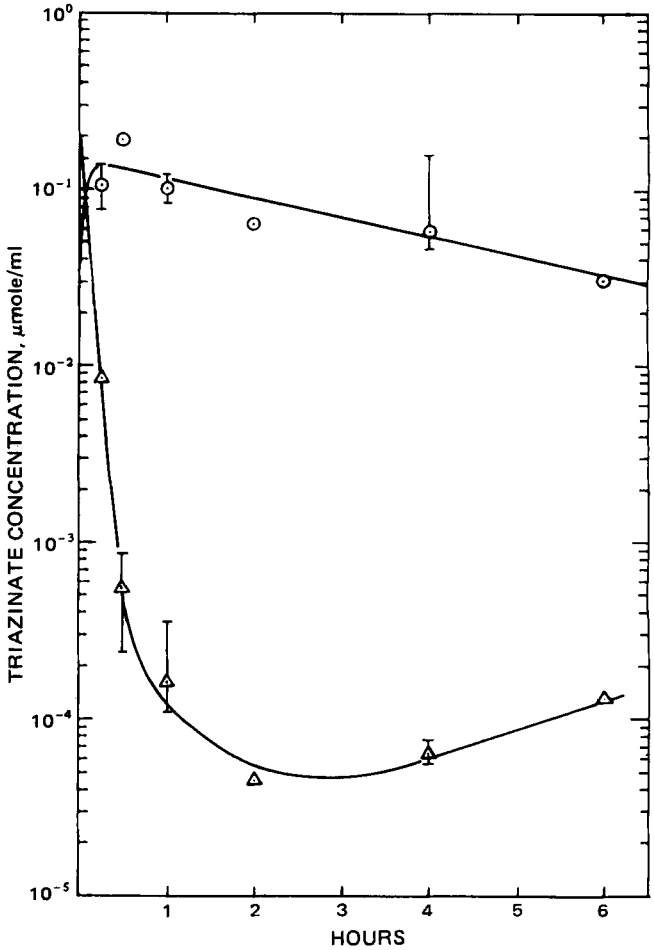


Figure 2—*Concentrations of triazinate in the peritoneal fluid and W-256 cells as a function of time following injections of 6 mg/kg ip (36 mg/m^2) in rats. Points represent experimental determinations (2). The solid lines represent the peritoneal fluid-forcing function (C_{pe}) and the model predictions of W-256 cell triazinate concentration (C_c). The peritoneal fluid triazinate concentration was fitted by the expression:*

$$C_{pe} = 0.203\, e^{-0.213t} + 4.31 \times 10^{-4}\, e^{-0.0224t} + 1.33 \times 10^{-5}\, e^{+0.00639t}$$

Other parameters are given in Table I. The bars represent ranges of the determinations. Key: (⊙) W-256 cells; (△) peritoneal fluid.

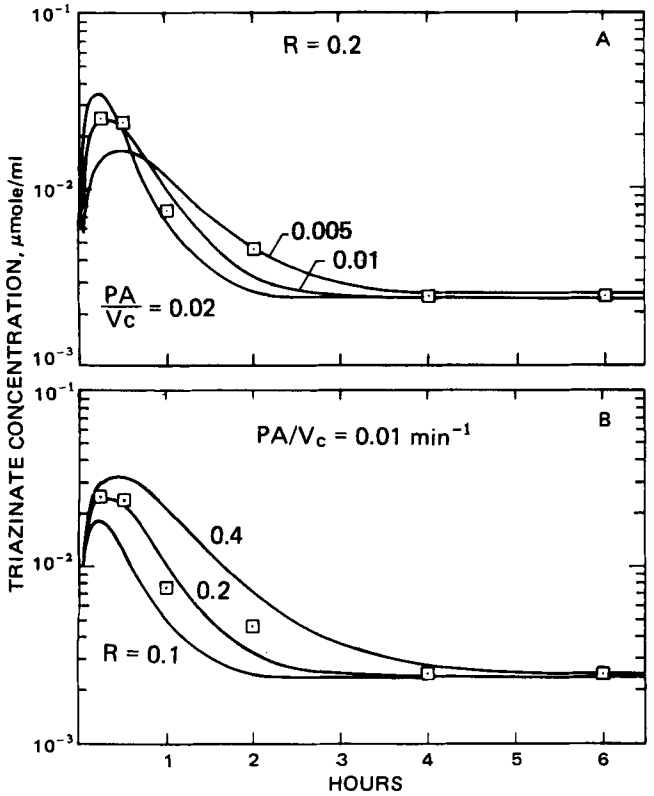


Figure 3—*Sensitivity of the model to changes in the parameters of (A) cell permeability (PA/V$_c$); and (B) partition coefficient (R) for the L-1210 cells in mice following injections of 12 mg/kg ip (36 mg/m^2). The points represent experimental determinations (2). The solid lines represent the model predictions of triazinate concentration in the L-1210 cells as a function of time.*

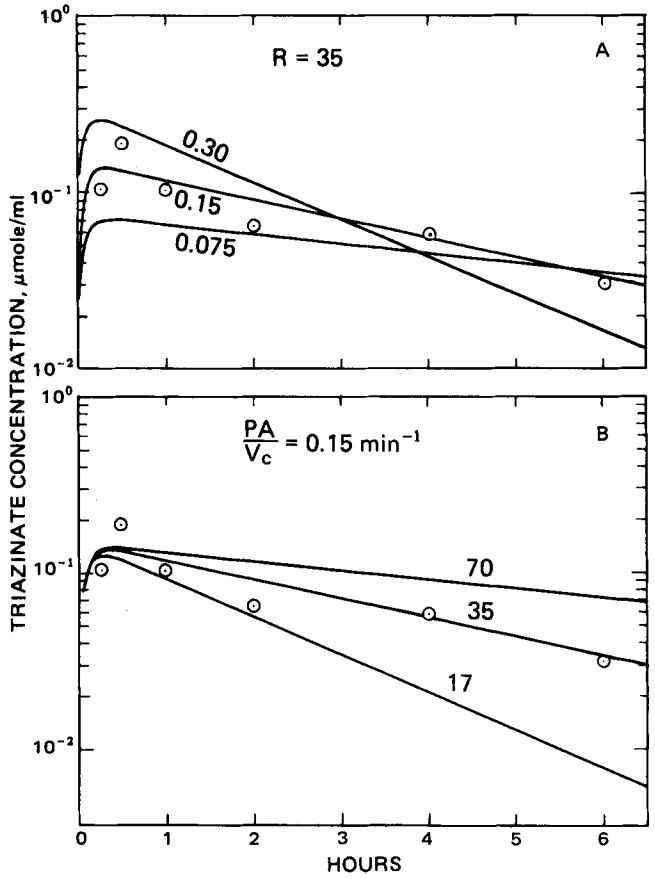


Figure 4—*Sensitivity of the model to changes in the parameters of (A) cell permeability (PA/V$_c$); and (B) partition coefficient (R) for the W-256 cells in rats following injections of 6 mg/kg ip (36 mg/m^2). The points represent experimental determinations (2). The solid lines represent the model predictions of triazinate concentration in the W-256 cells as a function of time.*

The total intracellular triazinate concentration in the tumor cells is the sum of free drug and drug bound to dihydrofolate reductase. The total tumor concentration in the model is related to the free drug concentration by the following relationship used previously for methotrexate (8):

$$C_c = C_c^F + \frac{aC_c^F}{\epsilon + C_c^F} \qquad \text{(Eq. 3)}$$

where a is the drug binding capacity of dihydrofolate reductase and ϵ is the dissociation constant of the drug–enzyme complex, both in micromoles per milliliter.

Values of the tumor cell permeabilities to triazinate, expressed as the quantity PA/V_c (min^{-1}), and the fluid–cell partition coefficients (R) are estimated by fitting Eq. 2 to the available data. These physicochemical parameters are summarized in Table I with the other model parameters. The system equations are solved by a Runge-Kutta routine (11) by computer to yield predictions of tumor cell triazinate concentration as a function of time.

RESULTS

Figures 1 and 2 show the peritoneal fluid and tumor cell concentrations of triazinate after injections of 36 mg/m^2 ip in mice and rats (12 and 6 mg/kg, respectively). The points represent the average of three individual determinations. The solid lines indicate the multiple exponential fits to the peritoneal fluid data and the model predictions of drug uptake by the L-1210 cells in mice (Fig. 1) and the W-256 cells in rats (Fig. 2). The average errors between model predictions and data are 16% for the L-1210 cells and 20% for the W-256 cells.

DISCUSSION

A pharmacokinetic model for triazinate uptake by L-1210 and W-256 ascites cells *in vivo* following a 36 mg/m^2 ip injection adequately simulates the observed concentration–time behavior in these cells. The model includes passive drug transport into the cells and binding of triazinate to intracellular dihydrofolate reductase. Values of binding constants and peritoneal fluid volumes are estimated from the literature (Table I), while the tumor cell volumes and other transport parameters (PA/V_c, R) are determined from the data (2).

A precise value for the dissociation constant (ϵ) for the triazinate–dihydrofolate reductase complex is not available. The value used here is that for methotrexate (8). Because triazinate is more tightly bound to dihydrofolate reductase than methotrexate, the ϵ for triazinate–dihydrofolate reductase should be smaller than ϵ for methotrexate–dihydrofolate reductase. However, since concentrations of free drug in the cells considered here are much greater than the magnitude of ϵ (of order 10^{-8} μmole/ml or less for triazinate), the expression for the amount of intracellular bound drug reduces to:

$$\frac{aC_c^F}{\epsilon + C_c^F} \rightarrow a \text{ (for } C_c^F \gg \epsilon) \qquad \text{(Eq. 4)}$$

Thus, a precise value of ϵ is not required.

The dramatic differences in triazinate uptake by the L-1210 and W-256 ascites cells are reflected in the model parameters determined from this study. The L-1210 cell permeability to drug (0.01 min^{-1}) is significantly smaller than that of the W-256 cells (0.15 min^{-1}), consistent with the observed difficulty of triazinate penetration in the leukemic cells compared with the W-256 cells (2, 3). While a partition coefficient of less than unity was determined for L-1210 cells, the W-256 partition coefficient is 35. These values also reflect the marked differences in drug accumulation in the two cell lines.

To assess the relative importance of permeability and partitioning in drug uptake and retention, a series of computer simulations was carried out. The effects of changing the transport parameters (PA/V_c) and distribution coefficients (R) are shown in Figs. 3 and 4 for the L-1210 cells and the W-256 cells, respectively. Each parameter is increased or decreased twofold while holding the remaining parameter constant. These simulations suggest that the cell permeability (PA/V_c) is the primary determinant of early accumulation of drug in tumor cells.

The preliminary model for triazinate pharmacokinetics presented here provides a logical basis for further investigation of the mechanism of triazinate transport and activity in different tumor lines.

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Disposition and Absolute Bioavailability of Furosemide in Healthy Males

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Abstract □ Furosemide (40 mg) was administered to 18 healthy adult males as an intravenous dose, an oral solution, and in tablet form. The pharmacokinetics of intravenous furosemide were studied, determining a total body clearance rate of 117.6 ± 41.3 ml/min and a harmonic mean half-life of 78 min. The mean absolute bioavailability determined by ratio of areas under the plasma–time curves was 64 and 71% for the solution and tablet, respectively. The mean absolute bioavailability determined by the ratio of urinary cumulative excretion data was 61 and 66% for the solution and tablet, respectively. The absolute bioavailabilities of furosemide determined with plasma and urine data were not significantly different. Thus, urine data alone may be used to establish bioavailability of furosemide. Inspection of plasma–time curves revealed secondary maxima in several subjects, suggesting enterohepatic cycling.

Keyphrases □ Furosemide—disposition and absolute bioavailability in healthy males □ Pharmacokinetics—disposition and absolute bioavailability of furosemide in healthy males □ Bioavailability—disposition, furosemide in healthy males

Furosemide is one of a series of anthranilic acid derivatives which is commonly used as a potent diuretic. Depending on the severity of clinical indication for its use, it is usually administered either orally or intravenously. Therefore, it is of interest to determine the bioavailability of oral preparations with respect to intravenous dosing.

Absolute bioavailability of furosemide has been studied previously by several investigators. Intravenous and oral doses of furosemide were administered previously to four subjects and the absolute bioavailability of tablets was determined to be 65%; the oral aqueous solution was 69% bioavailable (1). [^{35}S]Furosemide was administered orally as an aqueous solution to seven volunteers and intravenously to two different volunteers in another study (2). Comparison of the areas under the plasma curves across subjects determined the solution was 67% bioavailable. In a study with six volunteers (3), absolute bioavailability of oral furosemide (dosage form not identified) was found to be 49%. Eleven normal volunteers were studied (4); tablet and solution preparations were determined to be 69% bioavailable.

The present study was conducted to determine the absolute bioavailability of furosemide (40 mg) given in tablet form and as an oral solution to a large population of healthy males. In addition, the feasibility of using urinary excretion data alone to establish bioavailability was investigated. This would allow future bioavailability studies to be conducted without exposing subjects to numerous blood collections.

Absolute bioavailability of furosemide tablets and solution was established by both ratio of the areas under the plasma–time curves and ratio of cumulative excretion data. The disposition of furosemide given intravenously was also determined. Analysis of the resulting data strongly suggests enterohepatic cycling of furosemide.

EXPERIMENTAL

Subject Selection—Twenty-one healthy males, 20–31 years of age (mean 24) weighing between 61 and 83 kg (mean 71), who were in good physical condition as determined by physical examination, volunteered to participate in the study. Informed consent was obtained from each subject[1].

Study Design—An open Latin-square design was used to study 21 subjects divided into three groups of seven. Subjects were randomly as-

[1] The protocol has approval of the University of Texas at Austin Human Investigation Review Committee.

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