Biochemical Pharmacology, Vol. 46, No. 1, pp. 69–77, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
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METABOLISM AND PHARMACOKINETICS OF THE ANTI-HIV-1-SPECIFIC INHIBITOR [1-[2′,5′-BIS-*O*-(*TERT*-BUTYLDIMETHYLSILYL)-β-D-RIBOFURANOSYL]-3-*N*-METHYL-THYMINE]-3′-SPIRO-5″-(4″-AMINO-1″,2″-OXATHIOLE-2″,2″-DIOXIDE)

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(*Received* 10 *December* 1992; *accepted* 22 *March* 1993)

Abstract—[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-methyl-thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO-m^{3}T) is a potent, selective and specific inhibitor of human immunodeficiency virus type 1 replication *in vitro*. Uptake of TSAO-m^{3}T by human CEM cells is drug concentration-dependent and increased proportionally with increasing initial extracellular TSAO-m^{3}T concentrations up to 20 μg/mL. Within 6 hr of incubation, the cells were almost completely saturated with the test compound; further incubation up to 72 hr did not markedly increase the intracellular concentration of the compound. No intracellular metabolic conversion of TSAO-m^{3}T was observed in CEM, MT-4 or MOLT-4 cells. Upon intravenous bolus administration of TSAO-m^{3}T to mice at 0.75 mg/kg, TSAO-m^{3}T was rapidly cleared from the plasma in a mono-exponential manner (half-life: 22 min; distribution volume: 9.5 L/kg; total body clearance: 17.8 L/hr/kg). TSAO-m^{3}T mainly accumulated in the lungs, followed by the heart, kidney and liver. Significant amounts of different metabolites of TSAO-m^{3}T were detected in most tissues, the liver, kidney and spleen being the organs that showed the most extensive metabolism. The principal metabolites identified were TSAO-m^{3}T derivatives in which the *t*-butyldimethylsilyl moiety at C-2′ and/or C-5′ had been split off. The free base N^3-methylthymine was not detected.

Recently, several different structural classes of chemical compounds have been reported to show a specific and potent inhibitory effect on human immunodeficiency virus type 1 (HIV-1‡), but not HIV-2, simian immunodeficiency virus or other RNA or DNA viruses [1–8]. One of the most recently reported novel HIV-1-specific compounds is represented by [1-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO-T) (Fig. 1) [6, 9]. TSAO-T is inhibitory for HIV-1 replication in MT-4, CEM, MOLT-4 (clone 8), peripheral blood lymphocytes and monocyte/macrophages at a 50% effective concentration (EC_{50}) ranging between 0.017 and 0.076 μM [6, 9]. To demonstrate antiviral efficacy, the TSAO derivatives have to fulfill stringent structural requirements (i.e. concomitant presence of *tert*-butyldimethylsilyl groups at C-2′ and C-5′ of the ribose part of the molecule, as well as an intact 3′-spiro moiety in the (*R*) configuration [6, 9–13]. However, the thymine part of the TSAO-T molecule can be substituted by other pyrimidines (i.e. uracil, cytosine, 5-methylcytosine) and purines (i.e. adenine, hypoxanthine, xanthine) [6, 9, 13] without a marked decrease in antiviral efficacy. Substitution of an alkyl (i.e. methyl, ethyl) moiety at N^3 of the thymine ring results in TSAO derivatives that retain full antiviral potency but show markedly decreased cytotoxicity [6, 9, 13]. Consequently, the 3-alkyl-substituted TSAO-T derivatives possess the highest antiviral selectivity in cell culture. We have now investigated the cellular uptake, metabolism and pharmacokinetic properties of the N^3-methyl-substituted TSAO-T derivative TSAO-m^{3}T.

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‡ Abbreviations: TSAO-T, [1-[2′,5′,bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide); TSAO-m^{3}T, the N^3-methyl derivative of TSAO-T; HIV-1, human immunodeficiency virus type 1; dThd, thymidine; PBS, phosphate-buffered saline; PMEA, 9-(2-phosphonylmethoxyethyl)-adenine.

MATERIALS AND METHODS

Compounds. TSAO-T and TSAO-m^{3}T were synthesized according to a procedure that has been described recently [10–12]. Stock solutions were prepared at 20 mg/mL in dimethyl sulfoxide (100%), and further dilutions of the test compounds were made in 10% fetal calf serum-containing culture medium (for the cell culture experiments) or phosphate-buffered saline (PBS) (for the pharmacokinetic experiments).

Cells. Human T4 lymphocyte cells were kindly provided by Dr N. Yamamoto (MT-4, MOLT-4) (Tokyo Medical and Dental University School of Medicine, Tokyo, Japan) or obtained from the American Tissue Culture Collection (CEM) (Rockville, MD, U.S.A.). The cells were cultivated in

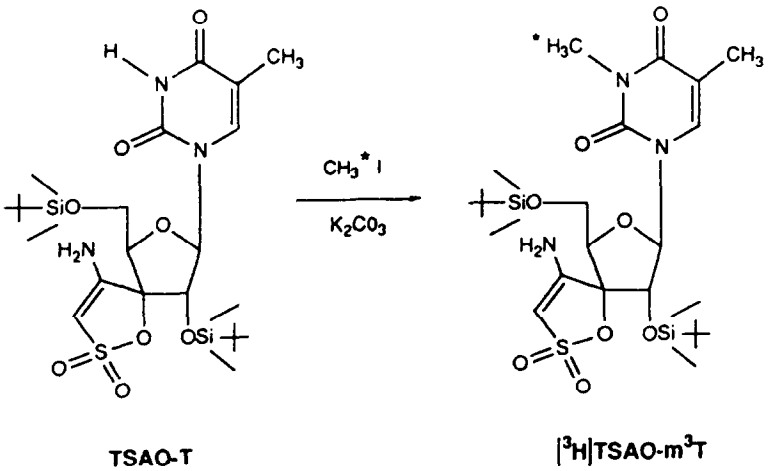

Fig. 1. Preparation of tritiated TSAO-m^{3}T. Reaction of TSAO-T with [^{3}H]methyl iodide in the presence of potassium carbonate gave the *N^3-methyl* tritiated [^{3}H]TSAO-m^{3}T in high yield.

RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 0.075% $NaHCO_3$.

Radiochemicals. Radiolabeled TSAO-m^{3}T (sp. radioact. 75 Ci/mmol), designated [^{3}H]TSAO-m^{3}T (^{3}H being part of the methyl group in the N^3-position), was synthesized according to a published procedure starting from TSAO-T [12, 13] (Fig. 1) and was obtained from Moravek Biochemicals Inc. (Brea, CA, U.S.A.). The radiochemical purity was higher than 99% as revealed by HPLC analysis on a Lichrocart Superspher 60 RP-8 column (Merck, Darmstadt, Germany).

Uptake and metabolism of [^{3}H]TSAO-m^{3}T in human CEM, MT-4 and MOLT-4 cells. The uptake and metabolism of different concentrations of radiolabeled [^{3}H]TSAO-m^{3}T was monitored as follows: CEM, MT-4 and MOLT-4 cells were seeded at 3×10^5 cells/mL in 6-mL culture flasks and incubated with different concentrations of [^{3}H]-TSAO-m^{3}T (i.e. 2, 5, 20 and 50 μg/mL for CEM cells, and 5 μg/mL for MT-4 and MOLT-4 cells). These concentrations were made by mixing a fixed amount of radiolabeled [^{3}H]TSAO-m^{3}T with varying concentrations of cold TSAO-m^{3}T. The total amount of radioactivity per cell culture was kept constant (1.66 μCi/mL). After 24 hr, the cells were centrifuged at 4°, washed three times with ice-cold medium [without serum, but containing unlabeled TSAO-m^{3}T (50 μg/mL)] and precipitated with 66% ice-cold methanol. After centrifugation at 10,000 rpm for 3 min, [^{3}H]TSAO-m^{3}T and its potential metabolites were quantitated in the supernatant by HPLC analysis using a Lichrocart Superspher 60 RP-8 column as described below.

In a second set of experiments, CEM cells were seeded at 3×10^5 cells/mL in 6-mL culture flasks and incubated with [^{3}H]TSAO-m^{3}T (at 5 μg/1.66 μCi/mL). At different time intervals (i.e. 0, 6, 16, 24, 48 and 72 hr), cells were centrifuged, washed and precipitated with 66% methanol whereafter [^{3}H]TSAO-m^{3}T was quantitated in the methanol-soluble cell material as described above.

Intracellular retention of [^{3}H]TSAO-m^{3}T in CEM cells after removal of the drug from the culture medium. CEM cells were seeded at 3×10^5 cells/mL and incubated with [^{3}H]TSAO-m^{3}T at 5 μg/1.66 μCi/mL for 24 hr. Then, the extracellular drug was carefully removed by centrifugation of the cells and washing the cell cultures three times with warm culture medium. At 0, 6, 24, 32 and 48 hr after removal of [^{3}H]TSAO-m^{3}T, CEM cell extracts were prepared and [^{3}H]TSAO-m^{3}T was determined by HPLC as described above.

Detection of [^{3}H]TSAO-m^{3}T in plasma. [^{3}H]-TSAO-m^{3}T was administered intravenously by bolus injection to female NMRI mice (weighing ~ 20 g) via the tail vein at a dose of 0.75 mg/kg in a total volume of 150 μL, containing 25% dimethyl sulfoxide, 12.5% ethanol and 62.5% PBS, and 18.8 μCi (15 μg) of [^{3}H]TSAO-m^{3}T. Mice were anesthetized with diethylether before blood was obtained by cardiac puncture (0.5–1 mL/mouse) at 10, 30, 60 or 90 min after administration of [^{3}H]-TSAO-m^{3}T. One mouse was taken for each time point. Two independent experiments were carried out. The blood was collected in heparinized tubes, and plasma was separated from the blood cells by centrifugation at 9000 *g* for 3 min and stored at −20° until analysis. Plasma extracts were prepared for analysis by adding 4 vol. of 100% methanol. The solutions were shaken vigorously for 10–15 min at 4°. Then, the samples were centrifuged at 9000 *g*, and the supernatants were further clarified by filtration (0.45 μm) and spiked with unlabeled TSAO-m^{3}T before being subjected to HPLC analysis. The chromatographic apparatus consisted of a LKB Pharmacia series 2248 pump, an 2157 automatic injector and a 2141 variable wavelength detector. Separation of [^{3}H]TSAO-m^{3}T and its metabolites from the plasma constituents was performed on a Lichrocart Superspher 60 RP-8 column (4 × 119 mm) using the following gradient elution protocol: 2 min 5% acetonitrile in H_2O; a linear gradient of 20 min to 90% acetonitrile in H_2O; 10 min 90% acetonitrile

in H_2O; 8 min linear gradient to 5% acetonitrile in H_2O and 5 min 5% acetonitrile in H_2O (equilibration). The flow rate was 1.0 mL/min. UV absorbance was measured at 273 nm. Radioactivity of the eluted fractions was determined by liquid scintillation counting. The retention times of the major radiolabeled metabolites were 2–3 min, 10–11 min, 13–14 min, 18–19 min and 24–25 min. Detection limit was 100 cpm (~0.80 ng TSAO-m^{3}T).

Tissue distribution of [^{3}H]TSAO-m^{3}T in NMRI mice. [^{3}H]TSAO-m^{3}T was administered intravenously to female NMRI mice (weighing ~ 20 g) via the tail vein at a total dose of 0.75 mg/kg (18.8 μCi/mouse). After 10, 30, 60 or 90 min, blood was collected by heart puncture, mice were killed by decapitation and organs were removed and flash-frozen in 1 mL of 100% methanol at −20°. Tissues were then frozen at −20° until further processed. Prior to extraction, tissues were weighed, homogenized and centrifuged at 9000 *g* for 5–10 min. The supernatant was then filtered (0.45 μm), spiked with unlabeled TSAO-m^{3}T and analysed by HPLC separation as described above.

Characterization of the [^{3}H]TSAO-m^{3}T metabolites. The retention times of the radiolabeled fractions were compared with the retention times of unlabeled TSAO-m^{3}T or TSAO-T, whether or not lacking the silyl moieties at either C-2′ or C-5′, or both, and N^3-methylthymine. The latter compound was obtained by acidic hydrolysis of N^3-methylthymidine (Sigma Chemical Co., St Louis, MO, U.S.A.) in the presence of 5 N HCl at 95°; the other compounds were synthesized following previously published procedures [10–12].

Pharmacokinetic calculations. [^{3}H]TSAO-m^{3}T concentrations in mouse plasma versus time after intravenous administration of [^{3}H]TSAO-m^{3}T to mice were fitted to mono-exponential functions ($C_t = C_0 \times e^{-K_e \cdot t}$) using least squares regression analysis. The elimination rate constant (K_e) ($0.0313 \times \text{min}^{-1}$) was derived from the slope of this linearized curve; the half-life ($T_{1/2}$) was calculated from the formula: $T_{1/2} = 0.693/K_e$. Distribution volume (V_D) and total body clearance (Cl_t) were calculated from the formulae: $V_D = \text{Dose}/C_0$ and $Cl_t = K_e \cdot V_D$. Average values for the pharmacokinetic parameters were calculated from the individual experiments.

Thymidine (dThd) phosphorylase assay. Purified dThd phosphorylase (Sigma) (1000 U/mL) was used in the assays. The change in absorbance was continuously monitored at the wavelength (274 nm) where the difference between dThd and its free base thymine was maximal. The reaction mixture consisted of 0.1 mM nucleoside (i.e. dThd, N^3-methylthymidine) or deprotected TSAO-T (i.e. without the silyl moieties at C-2′ and C-5′) in 100 μM sodium phosphate buffer pH 7.4 and enzyme at a final concentration of 0.2 U/mL when dThd was used as the substrate, and 2 U/mL when any of the other test compounds was used as the substrate. The conversion of nucleoside to free base was monitored by a decrease of absorbance as a function of time.

dThd kinase assay. The 70% $(NH_4)_2SO_4$ precipitate of a crude extract of CEM cells was submitted to DEAE column chromatography and subsequently to affinity column chromatography [using thymidine-3′-(4-aminophenylphosphate) linked to a carboxyhexyl-Sepharose carrier]. Synthesis of the affinity gel matrix was performed according to the procedure described by Kowal and Markus [14] and Whiteley *et al.* [15], and modified by Lee and Cheng [16]. The cytosol dThd kinase activity was measured in a standard reaction mixture containing 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 2 μM [*methyl*-^{3}H]dThd (sp. radioact.: 900 cpm/pmol), an appropriate amount of TSAO-T or TSAO-m^{3}T, and 5 μL of the purified enzyme in a total volume of 100 μL of 50 mM Tris–HCl pH 8.0. The reaction mixture was incubated at 37° for 15 and 30 min and the reaction terminated by spotting an aliquot (25 μL) onto DE-81 discs that were instantly immersed in ethanol (70%) and thoroughly washed in ethanol. The filters were then dried and assayed for radioactivity in a toluene-based scintillant.

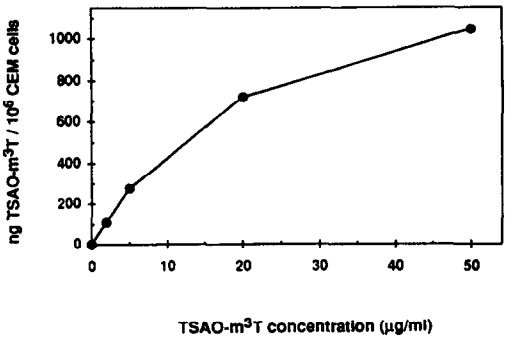

Fig. 2. Intracellular amounts of [^{3}H]TSAO-m^{3}T following incubation of CEM cells for 24 hr with different initial concentrations of [^{3}H]TSAO-m^{3}T.

RESULTS

Metabolism of [^{3}H]TSAO-m^{3}T in CEM, MT-4 and MOLT-4 cells

Upon incubation of CEM, MT-4 and MOLT-4 cells with [^{3}H]TSAO-m^{3}T at 5 μg/mL (8.25 μM), the intracellular [^{3}H]TSAO-m^{3}T levels measured after 24 hr were 365, 380 and 380 ng/10^6 cells (or 602, 627 and 627 pmol/10^6 cells, respectively). No metabolites of the parent compound were detected in any of the three cell lines examined. Uptake of [^{3}H]TSAO-m^{3}T was not inhibited when CEM cells were incubated with [^{3}H]TSAO-m^{3}T at 5 μg/mL in the presence of *S*-(*para*-nitrobenzyl)-6-thioinosine, an inhibitor of the nucleoside transport carrier (data not shown).

Uptake of [^{3}H]TSAO-m^{3}T in CEM cells as a function of different drug input concentrations

We then studied the uptake of [^{3}H]TSAO-m^{3}T in CEM cells as a function of different drug input concentrations. The intracellular concentrations of [^{3}H]TSAO-m^{3}T after 24 hr of incubation increased proportionally with the drug input concentration up to 10 μg/mL (Fig. 2). At higher initial TSAO-m^{3}T concentrations (i.e. 50 μg/mL), the increase in drug uptake slowed down. It should be mentioned here

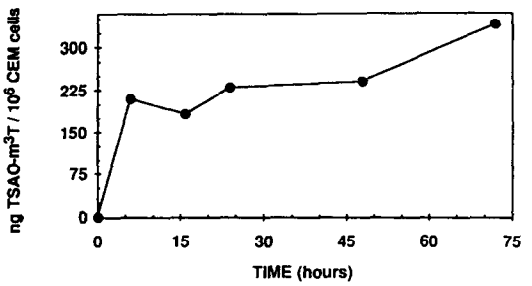

Fig. 3. Intracellular [^{3}H]TSAO-m^{3}T levels following incubation of CEM cells for different times with [^{3}H]-TSAO-m^{3}T at 5 μg/mL.

that this concentration is more than 1500-fold higher than the antivirally effective concentration obtained in human CEM and MT-4 cells (i.e. 0.030 μg/mL) [6, 9].

Uptake of [^{3}H]*TSAO-m^{3}T in CEM cells as a function of incubation time*

At an initial [^{3}H]TSAO-m^{3}T concentration of 5 μg/mL, uptake of the drug by the CEM cells was followed as a function of incubation time (Fig. 3). Within 6 hr of incubation [^{3}H]TSAO-m^{3}T had virtually reached its maximum intracellular concentration. Longer incubation times (i.e. 16, 24, 48 hr) did not result in a marked increase of the intracellular drug concentration. Even after 72 hr incubation intracellular [^{3}H]TSAO-m^{3}T levels were only slightly higher than the intracellular drug levels reached after 6 hr.

Retention of intracellular [^{3}H]*TSAO-m^{3}T upon removal of TSAO-m^{3}T from the culture medium*

CEM cells were incubated with [^{3}H]TSAO-m^{3}T at 5 μg/mL for a 24 hr period, after which the drug was removed from the extracellular medium. At 6 hr after removal of the drug only 33% of the intracellular drug remained intracellularly. Thereafter (at 24, 36 and 48 hr) intracellular [^{3}H]TSAO-m^{3}T levels

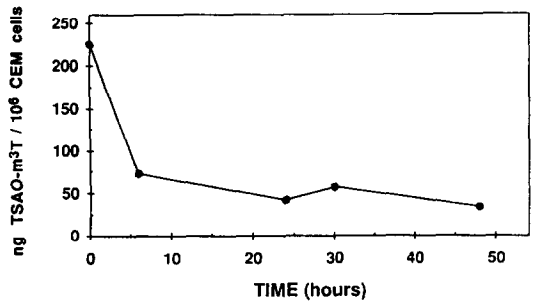

Fig. 4. Intracellular decay of [^{3}H]TSAO-m^{3}T in CEM cells upon preloading the cells with [^{3}H]TSAO-m^{3}T (5 μg/mL) for 24 hr followed by cultivation of the cells in culture medium in the absence of the compound.

Table 1. Pharmacokinetic parameters of [^{3}H]TSAO-m^{3}T in mice upon i.v. bolus injection at 0.75 mg TSAO-m^{3}T/kg

	$T_{1/2}$ (min)	V_D (L/kg)	Cl_t (L/hr/kg)
Individual values	18.0	8.4	19.3
	25.6	10.0	16.3
Mean	21.8	9.2	17.8

decreased slightly (Fig. 4). Based on these findings, the initial intracellular half-life of [^{3}H]TSAO-m^{3}T was estimated to be 4.5 hr. However, about 15% of the drug that was originally taken up was retained by the cells for at least 2 days.

Clearance of [^{3}H]*TSAO-m^{3}T from plasma in mice*

The decay of TSAO-m^{3}T in plasma was determined after i.v. bolus administration of 0.75 mg [^{3}H]TSAO-m^{3}T/kg to mice. Higher doses were not feasible due to the insolubility of the compound. Plasma drug concentrations versus time were fitted to a mono-exponential function to determine the elimination rate constant and plasma TSAO-m^{3}T half-life. The correlation coefficient of the linear regression curve was 0.984 (data not shown). TSAO-m^{3}T was rapidly cleared with a plasma half-life of 22.1 min and a total body clearance of 17.8 L/hr/kg (Table 1) (Fig. 5). However, it cannot be excluded that a much slower elimination phase occurs in plasma beyond the detection limit of our HPLC method.

Tissue distribution of [^{3}H]*TSAO-m^{3}T in mice*

[^{3}H]TSAO-m^{3}T levels in mouse tissues at 10 min after i.v. bolus injection of [^{3}H]TSAO-m^{3}T at 0.75 mg/kg (18.8 μCi) are shown in Table 2. Although the order of magnitude of the TSAO-m^{3}T levels in the individual tissues varied markedly from one experiment to another, the order of highest to lowest [^{3}H]TSAO-m^{3}T levels between the different organs was similar in three independent experiments. Within 10 min of injection high levels of [^{3}H]TSAO-m^{3}T were found in the lungs (265–5427 cpm/mg wet tissue). The heart and kidney contained 15-fold lower [^{3}H]TSAO-m^{3}T levels, whereas the liver,

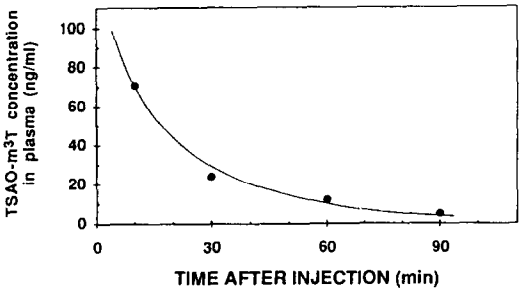

Fig. 5. Elimination curve of [^{3}H]TSAO-m^{3}T in mice upon i.v. bolus injection.

Table 2. Absolute tissue distribution of [^{3}H]TSAO-m^{3}T and relative [^{3}H]TSAO-m^{3}T concentration in tissue versus plasma at 10 min after i.v. bolus injection of [^{3}H]TSAO-m^{3}T

Tissue	Concentration of [^{3}H]TSAO-m^{3}T (cpm/mg wet tissue)				cpm [^{3}H]TSAO-m^{3}T tissue
	Individual experiments			Mean	cpm [^{3}H]TSAO-m^{3}T plasma
Lung	1858	265	5427	2517	44.8
Heart	108	142	232	161	3.23
Kidney	102	138	214	151	3.06
Liver	23	33	217	91	1.53
Spleen	52	60	128	80	1.56
Muscle	62	92	73	76	1.64
Thymus	56	58	72	62	1.30
Plasma	39	41	68	49	1.00
Blood	23	33	65	40	0.77
Brain	6	11	18	11	0.22

spleen, muscle and thymus contained 25–40-fold lower [^{3}H]TSAO-m^{3}T levels than the lungs. Brain tissue contained the lowest amounts of [^{3}H]TSAO-m^{3}T, but these levels were still significant and easily detectable (11 cpm/mg wet tissue) (Table 2).

When the ratios of the [^{3}H]TSAO-m^{3}T concentration in the different tissues to the [^{3}H]TSAO-m^{3}T concentration in the plasma were calculated from the values in Table 2, it became evident that within 10 min of exposure the lungs markedly accumulated the compound (44.8-fold), while the heart and kidneys showed only 3.06–3.26-fold higher TSAO-m^{3}T levels than those found in plasma. In contrast, brain tissue contained TSAO-m^{3}T levels that were 5-fold lower than plasma (Table 2).

When the [^{3}H]TSAO-m^{3}T levels in the different tissues were determined at 90 min versus 10 min after injection of the compound, a decay of the compound of 25-fold in plasma, 10–20-fold in the heart, kidney and lungs, 4–6-fold in the brain, muscle and thymus, but only 2-fold in the liver and spleen occurred (Table 3). The highest tissue versus plasma ratio of [^{3}H]TSAO-m^{3}T (at 90 min after injection) was found in the lungs (200-fold) as compared to 36- and 23-fold in the liver and spleen. Brain drug levels at 90 min after injection were equal to plasma [^{3}H]TSAO-m^{3}T levels (Table 3).

Metabolism of [^{3}H]*TSAO-m^{3}T in different tissues of mice*

[^{3}H]TSAO-m^{3}T was converted to varying radiolabeled metabolites depending on the tissue examined (Table 4). The lungs which accumulated the highest levels of [^{3}H]TSAO-m^{3}T poorly metabolized the compound. In fact, 93% of the radiolabel found in the lungs was unchanged parent compound. Only a small amount of a radiolabeled metabolite characterized by a retention time of 2–3 min (and

Table 3. Absolute tissue distribution of [^{3}H]TSAO-m^{3}T and relative [^{3}H]TSAO-m^{3}T concentration in tissue versus plasma at different time points after i.v. bolus injection of [^{3}H]TSAO-m^{3}T

Tissue	Concentration of [^{3}H]TSAO-m^{3}T (cpm/mg wet tissue)			
	Time after injection (min)			
	10	30	60	90
Lung	5427 (80)*	2522 (87)	2429 (209)	580 (200)
Heart	232 (3.4)	77 (2.6)	54 (4.7)	12 (4.1)
Kidney	214 (3.2)	81 (2.8)	39 (3.4)	16 (5.5)
Liver	217 (3.2)	147 (5.1)	176 (15)	105 (36)
Spleen	128 (1.9)	158 (5.4)	234 (20)	68 (23)
Muscle	73 (1.1)	56 (1.9)	34 (2.9)	16 (5.4)
Thymus	72 (1.1)	38 (1.3)	30 (2.6)	17 (5.7)
Plasma	68 (1.0)	29 (1.0)	12 (1.0)	2.9 (1.0)
Blood	65 (0.95)	27 (0.92)	ND†	7.6 (2.6)
Brain	18 (0.26)	8.7 (0.30)	13 (1.1)	2.9 (1.0)

* In parentheses are the tissue/plasma ratios for [^{3}H]TSAO-m^{3}T.
† Not determined.

Table 4. Relative tissue distribution of [^{3}H]TSAO-m^{3}T and its metabolites at 10 min after i.v. bolus injection of [^{3}H]TSAO-m^{3}T

	Percentage of total radioactivity*				
	Retention times (min)				
Organ	2/3	10/11	13/14	18/19	23/24
Lung	5 ± 6	1 ± 1	0.1 ± 0.1	2 ± 2	93 ± 9
Heart	14 ± 3	1 ± 2	0	6 ± 1	54 ± 46
Kidney	24 ± 8	9 ± 2	0	12 ± 2	56 ± 9
Liver	30 ± 11	28 ± 3	12 ± 4	11 ± 2	19 ± 17
Spleen	23 ± 7	1 ± 2	0	6 ± 5	70 ± 13
Muscle	16 ± 4	1 ± 2	0	7 ± 1	78 ± 5
Thymus	25 ± 5	3 ± 5	0	6 ± 5	66 ± 10
Plasma	28 ± 7	10 ± 3	8 ± 14	15 ± 13	40 ± 33
Blood	34 ± 9	4 ± 6	7 ± 12	14 ± 17	42 ± 34
Brain	52 ± 9	0	0	0	48 ± 9

* Data are the means ±SD for three independent experiments.

referred to as metabolite 2/3) could be detected. In contrast, in liver tissue at least four different radiolabeled metabolic products, designated metabolite 2/3, metabolite 10/11, metabolite 13/14 and metabolite 18/19 as based on their retention times by HPLC analysis, were detected. Both metabolite 2/3 and metabolite 10/11 were present in higher levels than the parent compound TSAO-m^{3}T. While shortly (10 min) after injection, the metabolites were present in the organs at lower levels than the parent compound, at later times (i.e. 30, 60 and 90 min), there was a marked decrease in [^{3}H]TSAO-m^{3}T and a concomitant increase in metabolite 2/3 (Table 5). Meanwhile, the metabolites 10/11, 13/14 and 18/19 almost completely disappeared (data not shown).

Characterization of the [^{3}H]TSAO-m^{3}T metabolites found in different tissues of mice

Attempts were made to characterize the different radiolabeled metabolites by comparison of their retention times in the HPLC analysis with those of different reference TSAO derivatives (Fig. 6). It should be mentioned that the retention times of the radiolabeled metabolite peaks are delayed 1–2 min when compared to the retention times of the unlabeled (cold) metabolite peaks due to the set-up of the UV recorder versus the fraction collector. Our examinations revealed that metabolite 18/19 co-elutes with the TSAO-m^{3}T derivatives lacking either the 2′-silyl moiety [designated TSAO-m^{3}T(2′-OH) or metabolite 18/19a] or 5′-silyl moiety [designated TSAO-m^{3}T(5′-OH) or metabolite 18/19b]. Metabolite 10/11 co-eluted with the TSAO-m^{3}T derivative lacking both the 2′- and 5′-silyl moieties of the molecule [designated TSAO-m^{3}T(2′,5′-OH) or deprotected TSAO-m^{3}T]. Metabolite 2/3 has been unable to be characterized so far. It was clearly different from the free base N^3-methylthymine (m^{3}T).

Affinity of TSAO-T and TSAO-m^{3}T for dThd kinase

Both dThd analogues TSAO-T and TSAO-m^{3}T were evaluated for their inhibitory effects on purified CEM cytosol dThd kinase. The compounds did not prove markedly inhibitory to dThd kinase at concentrations up to 1000 μM (data not shown).

Affinity of N^3-methylthymidine and deprotected TSAO-T for dThd phosphorylase

N^3-Methylthymidine and deprotected TSAO-T (lacking the silyl groups at both C-2′ and C-5′) were evaluated for their susceptibility to hydrolysis by dThd phosphorylase. While 100 μM dThd was completely hydrolysed by 0.2 U of the enzyme within 5 min at room temperature, N^3-methylthymidine and deprotected TSAO-T were completely resistant to phosphorolytic cleavage upon treatment with 2 U of dThd phosphorylase for 15 min (data not shown).

DISCUSSION

TSAO derivatives belong to a novel class of lipophilic nucleoside derivatives that are characterized by two peculiar structural characteristics: (i) the presence of a 3′-spiro moiety, and (ii) the presence of *t*-butyldimethylsilyl groups at both C-2′ and C-5′ of the pentose moiety. The silyl groups make the TSAO molecule lipophilic (partition coefficient in octanol/water: ⩾20) (data not shown). These silyl groups are quite firmly linked to the nucleoside. In fact, the compound is very stable in physiological solutions (i.e. PBS pH 7.2) at room temperature and no intracellular metabolites (besides the parent compound) were detected in at least three different human cell lines upon prolonged exposure of the cells to the compound.

The lipophilicity of TSAO-m^{3}T is most likely responsible for the rapid uptake of the compound into human T-lymphocytes. Indeed, most of the compound is taken up by CEM cells within 6 hr upon initial exposure of the compound to the cells. Moreover, the drug tends to accumulate intracellularly within this short period of time, since at least 20-fold higher levels of TSAO-m^{3}T are recovered intracellularly than initially added extracellularly.

Table 5. Disappearance of [³H]TSAO-m³T and appearance of metabolites as a function of time in several murine tissues

Tissue	Time after injection of [³H]TSAO-m³T (min)	Radioactivity (per cent of total)			
		Metabolite 2/3	Metabolite 10/11	Metabolite 18/19	Metabolite 23/24 (TSAO-m³T)
Lung	10	0.6	0.1	0.8	98
	30	1.3	0.2	0.6	98
	60	1.5	0.2	0.3	98
	90	3.9	0.7	0.5	95
Heart	10	12	0	7	81
	30	19	0	4	77
	60	38	0	0	62
	90	64	0	0	36
Kidney	10	17	10	13	59
	30	32	12	12	43
	60	43	18	15	24
	90	51	25	5	19
Liver*	10	17	26	9	39
	30	23	14	10	45
	60	19	14	5	57
	90	18	16	5	55
Spleen	10	15	0	0	85
	30	11	0	0	89
	60	12	0	0	88
	90	24	0	0	76
Muscle	10	20	0	7	73
	30	21	6	0	69
	60	33	6	4	58
	90	42	6	3	49
Thymus	10	25	0	0	75
	30	33	0	0	67
	60	48	0	0	52
	90	58	0	0	42
Plasma	10	31	8	9	52
	30	42	13	5	41
	60	64	13	4	19
	90	90	0	0	10
Brain	10	41	0	0	59
	30	42	11	0	47
	60	53	0	0	47
	90	81	0	0	19

* Only liver contained significant amounts of metabolite 13/14 (i.e. 9, 8, 6 and 5% after 10, 30, 60 and 90 min, respectively).

These observations may reflect specific distribution and binding of the compound, as well as an active transport mechanism, although it is unclear which transport system would be responsible for the uptake of TSAO derivatives by the cells. Also, the decay of intracellular TSAO-m³T levels in CEM cells is clearly biphasic: a rapid disappearance of the compound within the first 6 hr, followed by a very slow decay of the compound upon further incubation of the cells. Thus, the kinetics of uptake of the compound by the cells almost mirrors the kinetics of release of the compound from the cells into the extracellular medium.

The rapid uptake of [³H]TSAO-m³T by the cells *in vitro* is in agreement with our *in vivo* (mice) pharmacokinetic data and may explain the unusually high values found for the distribution value (V_D: 9.5 L/kg) and total body clearance (17.8 L/hr/kg). The V_D values are indicative of an extensive uptake of the compound by the different organs (tissues), and the high body clearance is indicative of a rapid renal clearance or rapid uptake by the tissues.

TSAO-m³T is rapidly cleared from the plasma when administered as an i.v. bolus, its half-life being 22 min. The plasma [³H]TSAO-m³T level has declined to the detection limit at 90 min after i.v. administration. Both the plasma half-life and body clearance of [³H]TSAO-m³T are higher than those reported for the 2′,3′-dideoxynucleoside analogues (i.e. 3′-azido-3′-deoxythymidine: $T_{1/2}$: 10–15 min; Cl_t: 1.9–2.6 L/hr/kg) [17–20] and acyclic nucleoside phosphonates (i.e. PMEA: $T_{1/2}$: 7–12 min; Cl_t: 1.2–2.4 L/hr/kg) [21]. These properties may well be related to the lipophilicity of the compound.

In contrast to the lack of metabolism of TSAO-m³T in cell cultures, TSAO-m³T seems to be metabolized *in vivo* since various metabolites were found in several murine tissues (i.e. liver, kidney,

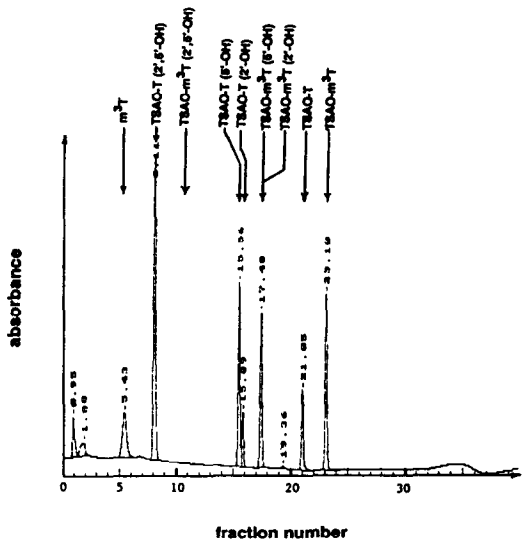

Fig. 6. HPLC profile of different TSAO derivatives. Abbreviations: m^3T, N^3-methylthymine; TSAO-m^3T(2′-OH) and TSAO-T(2′-OH), the TSAO-m^3T and TSAO-T derivatives, lacking the silyl group at C-2′; TSAO-m^3T(5′-OH) and TSAO-T(5′-OH), the TSAO-m^3T and TSAO-T derivatives, lacking the silyl group at C-5′; TSAO-m^3T(2′,5′-OH or deprotected TSAO-m^3T) and TSAO-T(2′,5′-OH or deprotected TSAO-T), the TSAO-m^3T and TSAO-T derivatives, without the silyl groups at both C-2′ and C-5′.

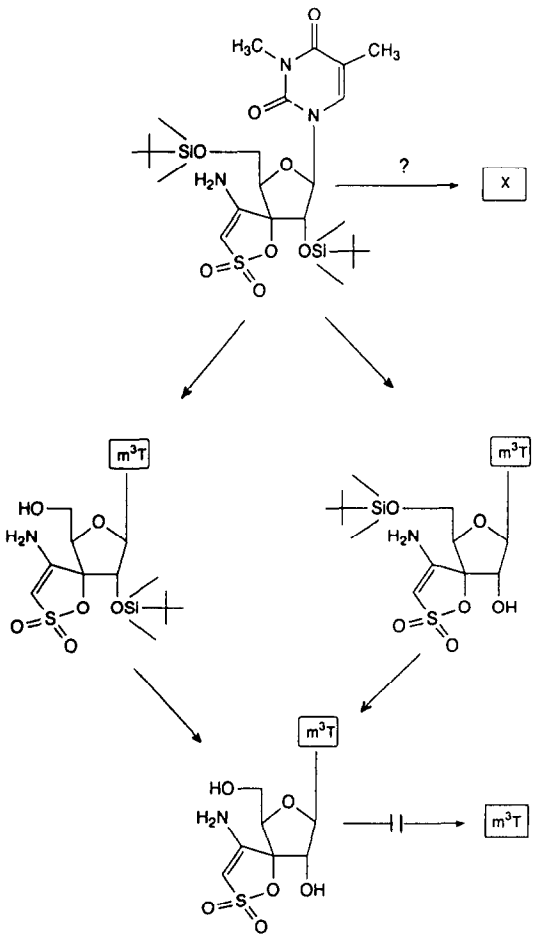

Fig. 7. Proposed metabolic conversion of [^{3}H]TSAO-m^3T in the liver.

spleen, thymus and heart). At least three metabolites have been unambiguously characterized, i.e. the TSAO-m^3T derivative lacking the silyl moiety at C-2′ (metabolite 18/19a), the TSAO-m^3T derivative lacking the silyl moiety at C-5′ (metabolite 18/19b), and the TSAO-m^3T derivative lacking the silyl groups at both C-2′ and C-5′ (metabolite 10/11). However, the eventual metabolite seems to be metabolite 2/3, which tends to accumulate progressively with time in different tissues. From the appearance/disappearance pattern of the different metabolites we may postulate that metabolite 2/3 is the end-product of a reaction sequence in which metabolites 18/19 and 10/11 probably act as intermediates or, alternatively, is progressively formed directly from the parent compound (Fig. 7). Metabolite 2/3 is clearly different from the free m^3T base. In fact, radiolabeled m^3T could not be detected in the different tissues. This is in agreement with our observations that N^3-methylthymidine and unprotected TSAO-T (lacking both silyl groups) are not substrates for purified dThd phosphorylase.

TSAO-m^3T accumulates in the lungs far more than in the other organs (i.e. heart, kidney). Tissue levels by far exceed those of plasma levels (i.e. an average of 45-fold for lungs versus 3.23- and 3.06-fold for heart and kidneys, respectively) at 10 min post administration to the mice. Moreover, the longer the time elapsed between compound administration and compound detection in the various organs, the greater the amounts of TSAO-m^3T accumulated in the lungs, liver and spleen versus plasma (Table 3). The reason for the preferential accumulation of TSAO-m^3T in the lungs is unclear.

In view of the neurological disorders associated with AIDS, it is of particular interest to note that the brain contains low but significant levels of TSAO-m^3T. Although TSAO-m^3T levels in the brain are low, they may suffice to achieve a therapeutic effect since TSAO-m^3T was found to be antivirally effective at a 50% inhibitory concentration as low as 30 ng/mL [6, 9]. Thus, our data indicate that TSAO-m^3T is able to cross the blood–brain barrier, and thus may be able to inhibit virus replication in the brain compartment.

Acknowledgements—This work was supported by grants from the Belgian "Fonds voor Geneeskundig Wetenschappelijk Onderzoek" (Krediet No. 3.0026.91) and the Belgian Geconcerteerde Onderzoeksacties (Krediet No. 91/94-2), by the NATO Collaborative Research Grant No. CRG 920777, by the Basic AIDS Research Programme of the European Community and by the Spanish Programma Nacional de Investigacion y Desarrollo Farmaceutico (Project No. FAR 88-0/606/1). We thank Ria Van Berwaer and Lizette van Berckelaer for excellent technical

assistance, and Christiane Callebaut for dedicated editorial help.

REFERENCES

1. Baba M, Tanaka H, De Clercq E, Pauwels R, Balzarini J, Schols D, Nakashima H, Perno CF, Walker RT and Miyasaka T, Highly specific inhibition of human immunodeficiency virus type 1 by a novel 6-substituted acyclouridine derivative. *Biochem Biophys Res Commun* **165**: 1375–1381, 1989.
2. Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ, Raeymaeckers A, Van Gelder J, Woestenborghs R, Heykants J, Schellekens K, Janssen MAC, De Clercq E and Janssen PAJ, Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. *Nature* **343**: 470–474, 1990.
3. Merluzzi VJ, Hargrave KD, Labadia M, Grozinger K, Skoog M, Wu JC, Shih C-K, Eckner K, Hattox S, Adams J, Rosenthal AS, Faanes R, Eckner RJ, Koup RA and Sullivan JL, Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science* **250**: 1411–1413, 1990.
4. Goldman ME, Nunberg JH, O'Brien JA, Quintero JC, Schleif WA, Freund KF, Gaul SL, Saari WS, Wai JS, Hoffman JM, Anderson PS, Hupe DJ, Emini EA and Stern AM, Pyridinone derivatives: specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. *Proc Natl Acad Sci USA* **88**: 6863–6867, 1991.
5. Romero DL, Busso M, Tan C-K, Reusser F, Palmer JR, Poppe SM, Aristoff PA, Downey KM, So AG, Resnick L and Tarpley WG, Nonnucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. *Proc Natl Acad Sci USA* **88**: 8806–8810, 1991.
6. Balzarini J, Pérez-Pérez M-J, San-Félix A, Schols D, Perno C-F, Vandamme A-M, Camarasa MJ and De Clercq E, 2′,5′-Bis-*O*-(tert-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)-pyrimidine (TSAO) nucleoside analogues: highly selective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase. *Proc Natl Acad Sci USA* **89**: 4392–4396, 1992.
7. Pauwels R, Andries K, Debyser Z, Van Daele P, Schols D, Vandamme A-M, Stoffels P, De Vreese K, Woestenborghs R, Janssen CGM, Anné J, Cauwenbergh G, Desmyter J, Heykants J, Janssen MAC, De Clercq E and Janssen PAJ, Potent and highly selective HIV-1 inhibition by a new series of α-anilino phenyl acetamide (α-APA) derivatives targeted at HIV-1 reverse transcriptase. *Proc Natl Acad Sci USA* **90**: 1711–1715, 1993.
8. Maass G, Immendoerfer U, Leser U, Pfaff E, Goody R and Koenig B, Viral resistance to the thiazolo-isoindolinones, a new class of non-nucleoside inhibitors of HIV-1 reverse transcriptase. *Abstracts of the HIV Drug-Resistance Workshop, Noordwijk, The Netherlands, 16–18 July 1992*, p. 29.
9. Balzarini J, Pérez-Pérez M-J, San-Félix A, Velázquez S, Camarasa M-J and De Clercq E, [2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO) derivatives of purine and pyrimidine nucleosides as potent and selective inhibitors of human immunodeficiency virus type 1. *Antimicrob Agents Chemother* **36**: 1073–1080, 1992.
10. Pérez-Pérez M-J, San-Félix A, Camarasa MJ, Balzarini J and De Clercq E, Synthesis of [1-[2′,5′-bis-*O*-(*t*-butyldimethylsilyl)-β-D-xylo and β-D-ribofuranosyl-thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (TSAO). A novel type of specific anti-HIV agents. *Tetrahedron Lett* **33**: 3029–3032, 1992.
11. Camarasa M-J, Pérez-Pérez M-J, San-Félix A, Balzarini J and De Clercq E, 3′-Spiro nucleosides, a new class of specific human immunodeficiency virus type 1 inhibitors: synthesis and antiviral activity of [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-xylo and -ribofuranose]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (TSAO) pyrimidine nucleosides. *J Med Chem* **35**: 2721–2727, 1992.
12. Pérez-Pérez M-J, San-Félix A, Balzarini J, De Clercq E and Camarasa M-J, TSAO analogues. Stereospecific synthesis and anti-HIV-1 activity of 1-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide]pyrimidine and pyrimidine-modified nucleosides. *J Med Chem* **35**: 2988–2995, 1992.
13. Balzarini J, Pérez-Pérez M-J, San-Félix A, Velázquez S, Camarasa M-J, Vandamme A-M, Karlsson A and De Clercq E, TSAO derivatives: a novel class of HIV-1-specific inhibitors. *Proceedings of the 3rd International Symposium on the Chemical Synthesis of Antibiotics and Related Microbial Products, Kloster Banz, Germany, 20–25 September 1992*. VCH, Weinheim, Germany.
14. Kowal EP and Markus G, Affinity chromatography of mammalian thymidine kinase. *FEBS Proc* **34**: 700, 1975.
15. Whiteley JM, Jerkunica I and Deits T, Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. Purification by affinity chromatography. *Biochemistry* **13**: 2044–2050, 1974.
16. Lee LS and Cheng YC, Human deoxythymidine kinase. I. Purification and general properties of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. *J Biol Chem* **251**: 2600–2604, 1976.
17. Doshi KJ, Gallo JM, Boudinot FD, Schinazi RF and Chu CK, Comparative pharmacokinetics of 3′-azido-3′-deoxythymidine (AZT) and 3′-azido-2′,3′-dideoxyuridine (AZddU) in mice. *Drug Metab Dispos* **17**: 590–594, 1989.
18. Eiseman JL, Bell MM, Bilello JA, Wetherell DL, MacAuley C and Yetter RA, Pharmacokinetics of 3′-azido-3′-deoxythymidine (AZT) in retrovirus-infected mice. *FASEB J* **2**: A913, 1988.
19. Kalin JR and Hill DL, Determination of 2′,3′-dideoxyadenosine, 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine in biological samples. *J Chromatogr* **431**: 184–191, 1988.
20. Kelley JA, Litterst CL, Roth JS, Vistica DT, Poplack DG, Cooney DA, Nadkarni M, Balis FM, Broder S and Johns DG, The disposition and metabolism of 2′,3′-dideoxycytidine, an *in vitro* inhibitor of human T-lymphotropic virus type III infectivity, in mice and monkeys. *Drug Metab Dispos* **15**: 595–601, 1987.
21. Naesens L, Balzarini J and De Clercq E, Pharmacokinetics in mice of the anti-retrovirus agent 9-(2-phosphonylmethoxyethyl)adenine. *Drug Metab Dispos* **20**: 747–752, 1992.