Direct Transport of 2′,3′-Didehydro-3′-deoxythymidine (D4T) and Its Ester Derivatives to the Cerebrospinal Fluid *via* the Nasal Mucous Membrane in Rats

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We investigated the absorption and transport of 2′,3′-didehydro-3′-deoxythymidine (D4T) and its ester prodrugs from the nasal cavity in rats. The absorption of D4T and its acetate (C2-D4T) was rapid and almost complete, although the hemi-succinate (Suc-D4T) was absorbed rather slowly; the plasma concentrations of the prodrug, Suc-D4T, and regenerated D4T remained unchanged throughout the experimental period (180 min). Concentrations in the cerebrospinal fluid (CSF) following intravenous (i.v.) and intranasal (i.n.) administration were also measured. After i.n. administration, drug concentrations were higher in the fraction derived from the subarachnoid space located close to the nasal mucosa than those in the fractions located far from the nasal cavity. This difference was not found following the i.v. administration of the drugs. Following nasal administration, the intact Suc-D4T was found in the CSF at a concentration higher than that of D4T, although transport of the intact prodrug to the CSF was not observed following i.v. administration. These results suggest that direct transport of the drugs from the nasal cavity to the CSF significantly contributes to the higher concentrations in CSF of D4T and/or its ester prodrugs, and indicate the possible value of nasal administration for the treatment of patients with AIDS dementia.

Key words cerebrospinal fluid (CSF); dideoxynucleoside; 2′,3′-didehydro-3′-deoxythymidine (D4T); AIDS; transport; prodrug

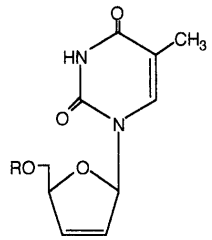
Human immunodeficiency virus (HIV) infection causes a variety of symptoms: the so-called acquired immunodeficiency syndrome (AIDS), opportunistic infections, cancers, and neurological disorders. AIDS dementia complex, a neurological syndrome characterized by abnormalities in cognition, motor performance, and behavior, is both a common and important cause of morbidity in patients in the advanced stage of HIV infection.[1] Since HIV itself is able to cross the brain capillary wall and infect the brain, it directly causes dementia.[2] Thus, delivery of anti-HIV agents to the brain is essential for effective pharmaceutical eradication of the virus from a patient. Although the transport of several anti-HIV agents to the central nervous system (CNS) has been reported,[3—6] the distribution of dideoxynucleosides into the interstitial fluid was minimal, as they showed slower penetration through the blood-brain barrier (BBB).[5] Attempts to increase the transport of dideoxynucleosides to the brain, including prodrugs[7—10] and combinations with transport inhibitors,[11—13] have been reported. However, the plasma concentration required for penetrating the BBB to obtain an effective concentration in the brain is still rather high and may cause systematic toxicity.

Since the nasal cavity is located close to the subarachnoid space, and there is evidence of a connection between the submucous bases of the nose with the perineural space,[14—16] there is potential for the direct transport of drugs to cerebrospinal fluid (CSF) from the nasal cavity.[17,18] Our previous study showed that zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) was absorbed well from the nasal cavity in rats, and a portion of the absorbed AZT reached the CSF without participation of the central bloodflow.[19] In this paper, we report the nasal absorption and transport to the CSF of 2′,3′-didehydro-3′-deoxythymidine (D4T), a potential anti-HIV agent with efficacy to AZT-resistant-HIV, and its ester prodrugs.

MATERIALS AND METHODS

D4T and Its Ester Prodrugs D4T was synthesized from thymidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the procedure of Horwitz *et al.*[20] The 5′-hydroxy position of D4T was acylated with acetic anhydride or succinic anhydride in dry pyridine to give the ester prodrugs of D4T: acetate (C2-D4T) and hemi-succinate (Suc-D4T). These compounds were purified over silica gel column chromatography and identified by NMR and MS. The purity of the samples was estimated to be more than 99% from the single peak in HPLC analysis. Chemical structures of D4T and its esters are shown in Fig. 1. All other chemicals were reagent or HPLC grade and were obtained commercially.

Animal Protocols Male Wistar rats (body weight 230—290 g) were anesthetized with 25% (w/v) of ethyl carbamate (4 ml/kg) by intraperitoneal injection, and the right femoral artery was cannulated with polyethylene tubing (i.d. 0.28 mm, o.d. 0.61 mm) for blood sampling.



-R	Compound
-H	D4T
$-COCH_3$	Acetate(C2-D4T)
$-CO(CH_2)_2COOH$	Succinate(Suc-D4T)

Fig. 1. Chemical Structures of D4T and Its Ester Prodrugs

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Intranasal (i.n.) Administration Studies A surgical operation to the esophagus and trachea was carried out as described by Hussain *et al.*[21] The trachea was cannulated with polyethylene tubing (i.d. 1.6 mm, o.d. 2.6 mm). A silicone tube (i.d. 1.0 mm, o.d. 2.0 mm) was inserted from the esophagus to the posterior part of the nasal cavity. The nasopalatine was closed with an adhesive (Aron Alpha, Toa Gousei Co., Japan). Each drug was administered at a dose of 44.6 μmol/kg into the nasal cavity (10 mg/kg, D4T equivalent) through a nostril using a micropipet (100 μl/rat). D4T was prepared as a solution and the prodrugs were suspended in pH 7.4 isotonic phosphate buffer at a concentration of 133.8 μmol/ml. Following the intranasal (i.n.) administration, CSF samples were collected at 15, 30, 60, or 180 min. Only one sampling was performed on each rat, thus, five animals were used for one time point.

Intravenous (i.v.) Administration Studies The same surgical operation described above was performed on these rats to cancel out the effects of the operation and tubing. Drugs were injected as a saline solution into the jugular vein. The dose of D4T and Suc-D4T was 44.6 μmol/kg and that of C2-D4T was 22.3 μmol/kg because of its poor solubility in saline. Blood samples were collected at 5, 10, 15, 30, 45, 60, 120 and 180 min following the administration. Besides this collection from the rats for the blood concentration measurement, a CSF sample following i.v. administration was collected at 15 or 180 min from independent rats.

Collection of CSF The cisternal puncture technique reported by Chou and Levy[22] was used to collect CSF, employing the sharp end of a 25-gauge needle which had a 1.5 m length of fine polyethylene tubing (SP-31, i.d. 0.5 mm, Natume Co., Japan) attached to a disposable syringe. This fine tubing made it possible for CSF to be separated as it was collected. Collection was terminated as soon as blood appeared in the tubing. In all studies, the CSF sample was used only if more than 150 μl was collected without any blood contamination. The CSF was separated into three fractions, as shown in Fig. 2. The first 50 μl of the CSF was referred to as fraction R, the next 50 μl as fraction M and last 50 μl as fraction F, respectively. Each fraction was stored at −40 °C until quantitative analysis.

Stability of Prodrugs in CSF Rat CSF was collected from the control animals according to the method described above. CSF samples without any blood contamination were stored at −40 °C until use. The chemical stability of the prodrugs was reported previously,[23] and showed that they are quite stable under physiological conditions. The enzymatic hydrolysis rates were determined in the presence of CSF at 37 °C. The hydrolysis was initiated by adding the stock solution (4 μl of pH 7.4 isotonic phosphate buffer solution) to preincubated CSF (300 μl) to give an initial concentration of 2×10^{-5} M, the lowest concentration suitable for quantitative analysis of the compounds, and at 4×10^{-5} M. A 50 μl portion of the reaction mixture was collected periodically and deproteinized with the same volume of methanol. The resulting sample was injected onto the HPLC column and concentrations of the prodrug and D4T were determined. The reactivities to enzymatic hydrolysis were evaluated as pseudo-first-order rate constants. The constants were obtained from the slopes of semilogarithmic plots of 2×10^{-5} M minus the regenerated D4T concentration, which was consistent with the prodrug concentration in the same sample, against time. The enzymatic reaction was not saturated at the higher substrate concentration (4×10^{-5} M).

Stability of Prodrugs in Nasal Tissue Homogenate Following ethyl carbamate overdose and exsanguination, the rat nasal tissue was removed. Respiratory mucosa, dissected free from the bone, was removed from the naso- and maxilloturbinates, the anterior two-thirds of the lateral walls, and the ventral half of the nasal septum. Olfactory mucosa was removed from the ethmoturbinates, the posterior one-third of the lateral walls, and the posterior two-thirds of the roof of the nasal cavity. The tissues were homogenized in phosphate buffer (pH 7.4) using a coaxial driven Teflon pestle and glass mortar. The homogenates were then centrifuged at 1000×***g*** for 10 min and aliquots were stored at −40 °C until use. The enzymatic hydrolysis rates were determined in the presence of the homogenate (4% (w/v)) at 37 °C, using the same method as that for the CSF.

Analytical Procedure The presence of drugs in plasma, CSF and nasal tissue homogenate were determined by an HPLC system which was composed of a Shimadzu LC-10AS pump, a Shimadzu SPD-10A UV detector, a Reodyne 7125 injector and a reversed-phase column (Lichrospher 100 RP-18e, 250×4 mm). The mobile phase was a mixture of water : methanol : acetic acid at 84 : 16 : 0.2 for D4T and at 80 : 20 :

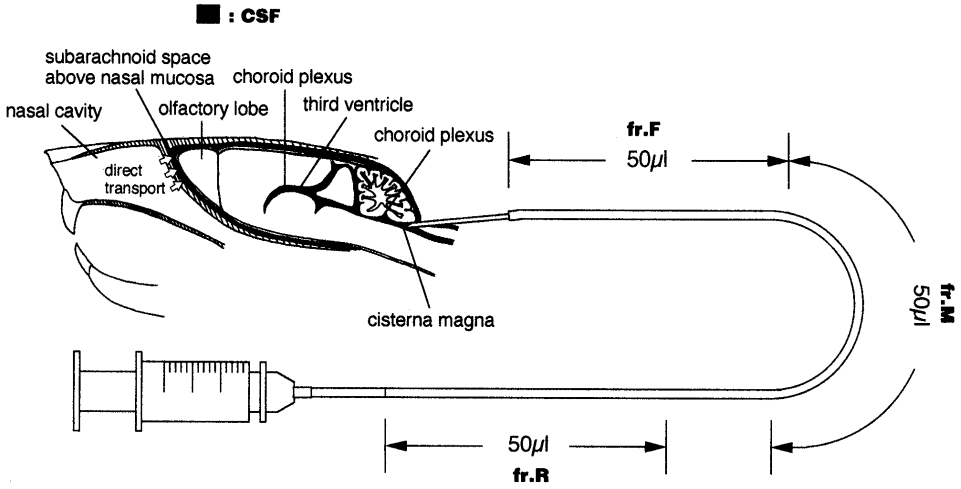

Fig. 2. The Method of Collection (Cisternal Puncture) and the Division of Samples in Rats

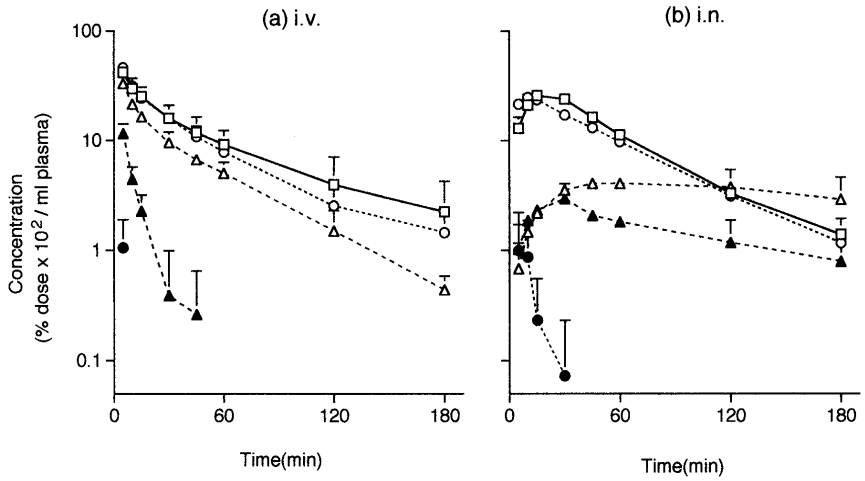

Fig. 3. Plasma Concentration *vs* Time Profiles of D4T and Its Prodrugs Following i.v. (a) and i.n. (b) Administration

□, D4T; ●, C2-D4T (detected as C2-D4T); ○, C2-D4T (detected as D4T) ; ▲, Suc-D4T (detected as Suc-D4T) ;△, Suc-D4T (detected as D4T). (n=3, mean±S.D.).

0.2 for C2-D4T and Suc-D4T. The wavelength of the detection was 265 nm. The eluant was pumped at a rate of 1.0 ml/min with a column temperature of 40 °C. The approximate retention times of D4T, C2-D4T, Suc-D4T and an internal standard, 1-(2-deoxy-3,5-epoxy-β-D-threo-pentofuranosyl) thymine, were 4.2, 10.0, 4.9, and 5.4 min, respectively. The preparation of plasma samples was the same as reported previously.[23]

Pharmacokinetic Analysis The estimation of the area under the curve (*AUC*) from plots of the plasma D4T concentration *versus* time was calculated using the trapezoidal rule. The area from the last data point to infinity was estimated by the extrapolation elimination constant of the terminal phase.

RESULTS AND DISCUSSION

Absorption from the Nasal Cavity to the Central Blood-Flow Transport to the central blood flow following i.v. and i.n. administration was measured to determine how a drug reaches the CSF. Figure 3 shows the plasma concentration *versus* time profiles of D4T and its esters following i.v.: a and i.n.: b administration. Absorption of D4T from the nasal cavity was rapid and almost complete, as indicated by the absolute bioavailability of 98%. Because of its low water solubility, the i.v. administration of C2-D4T was performed at only half the dose (22.3 μmol/kg), and the results together with those of the full dose (44.6 μmol/kg) i.n. administration are represented as a dose-normalized % in Fig. 3. The conversion of C2-D4T to D4T must be very fast by either i.v. or i.n. administration, since the prodrug was only detected at a very low concentration (Fig. 3a, b; ●) immediately following administration, and the elimination rate of regenerated D4T after the administration of C2-D4T was almost the same as that following D4T administration (*i.e.* $t_{1/2}$ of D4T; i.v. 45 min, i.n. 40 min, C2-D4T; i.v. 49 min, i.n. 40 min). The absorption of C2-D4T from the nasal cavity was also rapid, with a bioavailability (*AUC* of regenerated D4T following i.n. administration of C2-D4T/*AUC* of D4T following i.v. administration of D4T) of 90.5%. The plasma concentration *versus* time profiles of Suc-D4T and D4T following Suc-D4T administration (Fig. 3; detected as Suc-D4T: ▲, D4T: △) were quite different from those of either C2-D4T or D4T.

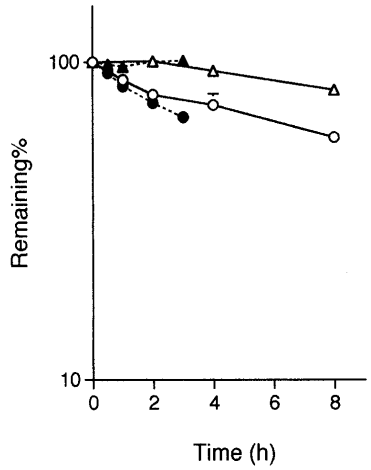

Fig. 4. Enzymatic Stability of D4T Esters in the Nasal Tissue Homogenate (Closed Symbol) and in the CSF (Open Symbol)

○, ●, C2-D4T; △, ▲, Suc-D4T. Vertical bars indicate standard deviations (n=3).

The intact prodrug was detected for 45 min following i.v. administration of Suc-D4T and the bioavailability of D4T calculated from the data up to 180 min was about 60%. This low bioavailability may be partly attributable to the intact prodrug being eliminated before its conversion to D4T. Following i.n. administration, the concentrations of both Suc-D4T and D4T remained constant throughout the experimental period. This high and continuous concentration of the intact prodrug (Suc-D4T) may contribute to its lower enzymatic reactivity. Figure 4 shows the enzymatic reactivity of C2-D4T and Suc-D4T in the rat nasal mucosa homogenate (closed symbol). A large difference in the hydrolysis rate constants was observed between C2-D4T (5.5×10^{-2} min^{-1}) and Suc-D4T ($<1.0\times10^{-4}$ min^{-1}). This result is consistent with the previous finding that the enzymatic reactivity of Suc-D4T is much lower than those of neutral esters, including C2-D4T.[23]

Transport from the Nasal Cavity to the CSF D4T concentrations in the CSF after i.v. and i.n. administration of the drug are shown in Fig. 5. The concentration in fraction F at 15 min after the i.n. administration (data range from 49.45 to 10.88 μM) was remarkably high and the CSF/plasma concentration ratio was more than 1.0. Since the CSF samples were

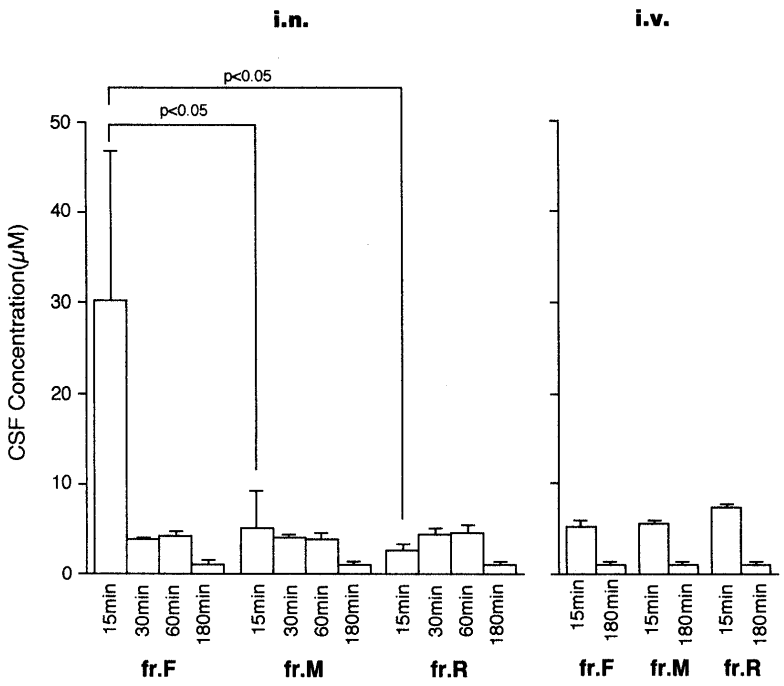

Fig. 5. CSF Concentration of D4T Following i.n. and i.v. Administration of D4T. Vertical bars indicate standard deviations ($n=5$).

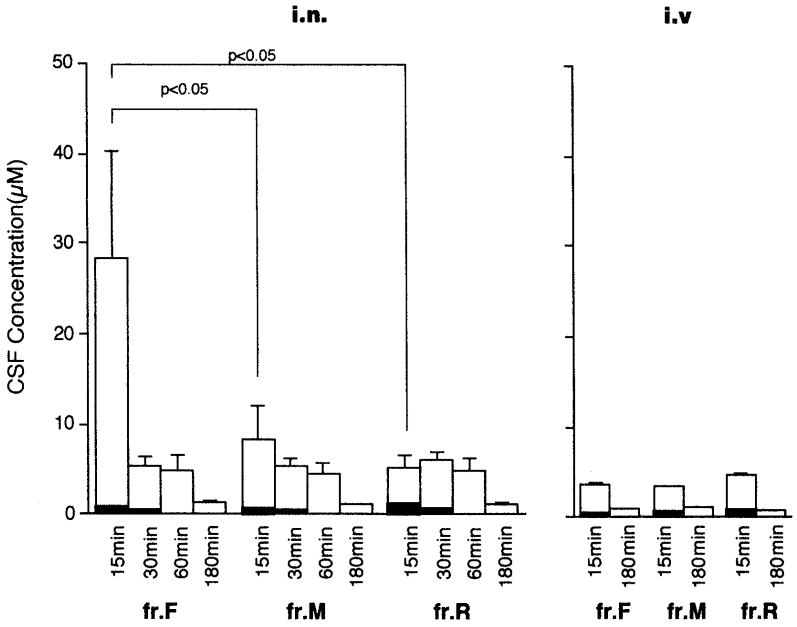

Fig. 6. CSF Concentration of D4T and C2-D4T Following i.n. and i.v. Administration of the Prodrug

Closed bars indicate C2-D4T concentration: ■. Open bars indicate D4T concentration regenerated from C2-D4T: □.Vertical bars indicate standard deviations ($n=5$).

withdrawn from the cisterna magna, which is located far from the subarachnoid space above the nasal mucosa (seen in Fig. 2), the higher concentration in the later fraction (fr. F) during the absorption period suggests the direct transport of the drug from the nasal cavity to the CSF. This gradient in CSF concentrations was not observed after the i.v. administration of D4T. Similar observations and their relation to the direct transport from nasal mucosa to the CSF have been reported by several researchers.[24,25] Sakane *et al.* have reported that sulfonamides are transported directly from nasal cavity to CSF, and that the direct pathway depends on the lipophilicity of the drugs.[17] In our study, though the lipophilicity of AZT ($\log PC=-0.31$)[26] was larger than that of D4T ($\log PC=-1.54$),[23] the CSF/plasma concentration ratio of AZT (0.361 ± 0.276) at 15 min following i.n. administration was smaller than that of D4T (1.098 ± 0.585). It is not clear what mechanism influenced the difference; there may be an influx and efflux into the CSF involved in the active transport system.

CSF is produced from the choroid plexus at the ventricle, and thereafter flows in the subarachnoid space, which would mean that the subarachnoid space above the nasal mucosa (*i.e.* fr. F) is located far from the choroid plexus. The location of fr. F in the CSF may suggest that the drug directly transported into fr. F was rapidly excreted by the bulk flow.[27]

The concentration of D4T in fr. F (data range from 39.16

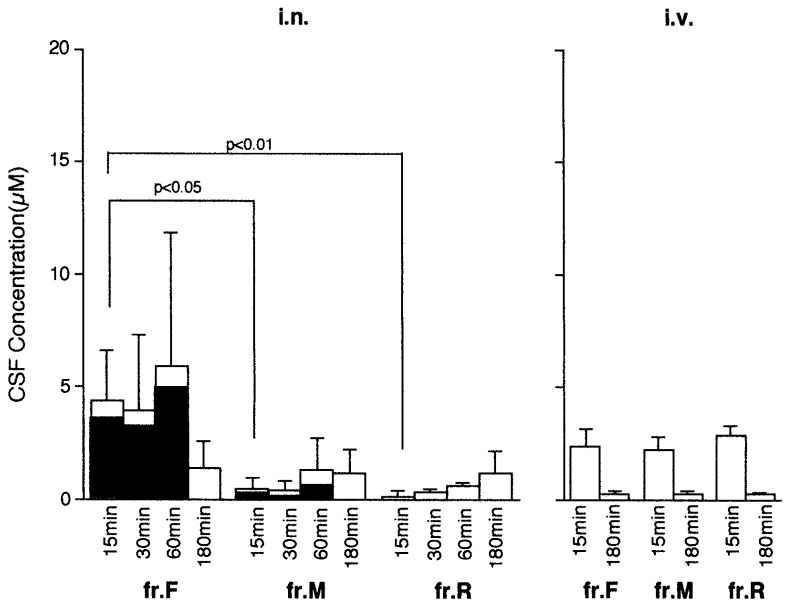

Fig. 7. CSF Concentration of D4T and Suc-D4T Following i.n. and i.v. Administration of the Prodrug

Closed bars indicate Suc-D4T concentration: ■. Open bars indicate D4T concentration regenerated from Suc-D4T: □. Vertical bars indicate standard deviations ($n=5$).

to 12.11 μM) was also high at 15 min after the i.n. administration of C2-D4T, but this was not observed following the i.v. administration of the same prodrug (Fig. 6). Intact prodrug (closed columns) was detected up to 30 min after the i.n. administration, although the concentration was much lower than that of regenerated D4T. Since C2-D4T is labile for enzymatic hydrolysis, as shown in Fig. 4, a large part of it in the nasal cavity may be hydrolyzed to D4T before or during transportation to the CSF. The stability of the prodrugs in the CSF is shown in Fig. 4 (open symbol). Since less than 10% of the prodrugs were degraded at 60 min, both seemed to be stable in the CSF during the period of the absorption experiment. The CSF concentrations following the administration of Suc-D4T (Fig. 7) were higher in fr. F than those in fr. M or fr. R up to 60 min after i.n. administration, although this was not found with i.v. administration. The gradual increase in the concentrations in fr. F could be attributable to the slower absorption of Suc-D4T from the nasal cavity, as seen in Fig. 3b. As mentioned above, Suc-D4T is expected to be stable in brain parenchyma, which may lead to a low concentration of regenerated D4T in the brain parenchyma cell. However, this continuous transfer pattern into the CSF is favorable for time-dependent drugs such as D4T. Following the i.n. administration of Suc-D4T, a large part of the CSF concentration in fractions F and M was the intact prodrug (indicated by closed columns in Fig. 7). This phenomenon strongly suggests that Suc-D4T is directly transported to the CSF, since the intact prodrug was not detected in these fractions following i.v. administration.

The species difference is very important for drug delivery to the CNS by nasal application. The CSF turnover rate in rats (0.75%/min) is faster when compared with that of human (0.38%/min).[28] Moreover, the relationship of anatomical location between the nose and brain are different in rats and humans, which suggests that the CSF above the nasal mucosa is not always located far from the choroid plexus in human.

D4T and C2-D4T are well absorbed from the nasal cavity of rats. Drug concentrations in the CSF following i.n. administration were higher than those obtained by i.v. The CSF concentration in the fraction derived from the subarachnoid space close to the nasal mucosa (fr. F) was higher than that in the other fractions obtained during the absorption period. Suc-D4T administered in the nasal cavity reached the CSF in an intact form, but that administered by i.v. did not. This phenomenon indicates that this hydrophilic compound (log PC = −3.63)[29] is mainly transported to the CSF *via* a non-systematic route.

Recently, Takasawa *et al.* have reported that CSF concentration cannot be used as an index of the brain tissue concentration of AZT and 2′,3′-dideoxyinosine (DDI), and the restricted distribution of these drugs is responsible for the efflux transport process across the BBB using the distributed model in rats.[30] On the other hand, Yang *et al.* have demonstrated that the direct intracerebroventricular administration of D4T enhances its brain delivery, and D4T exhibits a more favorable penetration into the brain than AZT, using freely-moving rat.[31] Additionally, Sakane *et al.* reported that the brain uptake clearance of 5-fluorouracil following i.n. infusion was larger than that following i.v. infusion.[32] These reports suggest that a drug can be delivered to the brain through the direct pathway. Although the brain tissue concentrations of the drugs were not determined, our results suggest the direct transport of D4T and/or its ester prodrugs from the nasal cavity to the CSF, indicating the usefulness of this route for the treatment of AIDS dementia.

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