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Cyclosaligenyl-2′,3′-didehydro-2′,3′-dideoxythymidine Monophosphate: Efficient Intracellular Delivery of d4TMP

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ABSTRACT

Cyclosaligenyl-2′,3′-didehydro-2′,3′-dideoxythymidine-5′-monophosphate (cycloSal-d4TMP) is a potent and selective inhibitor of human immunodeficiency virus replication in cell culture and differs from other nucleotide prodrug approaches in that it is designed to selectively deliver the nucleotide 5′-monophosphate by a controlled, chemically induced hydrolysis. Its antiviral efficacy in cell culture is at least as good as, if not superior to, that of d4T. CycloSal-d4TMP was found to lead to the efficient intracellular release of d4TMP in a variety of cell lines, including both wild-type CEM and thymidine kinase-deficient CEM/TK$^-$ cells. Under similar experimental conditions, exposure of CEM/TK$^-$ cells to d4T failed to result in significant d4TTP levels. The intracellular conversion of cycloSal-d4TMP proved to be both time and dose dependent. The half-life of d4TTP generated intracellularly from d4T- or cycloSal-d4TMP-treated CEM cells was ~3.5 h, and the intracellular ratios of d4TTP/d4TMP in cells exposed to cycloSal-d4TMP gradually increased from 1 to 3.4 upon prolonged incubation. Radiolabeled cycloSal-d4TMP could be separated as its two R_p and S_p diastereomers on high-performance liquid chromatography. The R_p diastereomer of cycloSal-d4TMP was 3- to 7-fold more efficient in releasing d4TMP and generating d4TTP than the S_p cycloSal-d4TMP diastereomer. This correlated well with the 5-fold more pronounced antiviral activity of the R_p diastereomer versus the S_p diastereomer. d4TMP is a poor substrate for the cytosolic 5′(3′)-deoxyribonucleotidase (V_{max}/K_m for d4TMP: 0.08 of V_{max}/K_m for dTMP) and is only slowly hydrolyzed to d4T. This contributes to the efficient conversion of the prodrug of d4TTP.

A variety of nucleoside analogs are endowed with anticancer or antiviral activity. They are not biologically active as such, but they need to be converted (phosphorylated) to the 5′-mono-, 5′-di-, or 5′-triphosphate forms to eventually reach their molecular target (enzyme) for cytostatic or antiviral activity. For many nucleoside analogs, the first phosphorylation step is rate limiting, and phosphorylating enzymes often have a poor affinity for the unnatural nucleoside analogs (Balzarini et al., 1988, 1989; Starnes and Cheng, 1987; Johnson and Fridland, 1989). To circumvent this first phosphorylation (activation) step, a variety of prodrug approaches have been designed in attempts to release the nucleoside 5′-monophosphate directly into the intact cells. If successful, such approach should lead to a more pronounced cytostatic or antiviral activity, and, indeed, a number of prodrug attempts have proved successful (for an overview, see Meier, 1998). Given the fact that 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) is used in the clinical setting for the treatment of human immunodeficiency virus (HIV)-infected individuals, and considering the unfavorable pharmacological properties of d4T [the affinity of d4T for cytosolic thymidine kinase (TK) is rather poor (K_i/K_m ~100; Balzarini et al., 1989)], d4T can be envisaged as an obvious candidate drug in a prodrug approach to directly deliver 2′,3′-didehydro-2′,3′-dideoxythymidine-5′-monophosphate (d4TMP) inside intact cells.

Here, we report on the intracellular metabolism of a novel type of prodrug molecule, designated cyclosaligenyl-2′,3′-didehydro-2′,3′-dideoxythymidine-5′-monophosphate (cycloSal-d4TMP; Fig. 1). This neutral monophosphate prodrug triester of the anti-HIV drug d4T has recently been shown to have improved antiviral potential against HIV in CEM and

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ABBREVIATIONS: HIV, human immunodeficiency virus; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; TK, thymidine kinase; d4TMP, 2′,3′-didehydro-2′,3′-dideoxythymidine-5′-monophosphate; cycloSal-d4TMP, cyclosaligenyl-2′,3′-didehydro-2′,3′-dideoxythymidine-5′-monophosphate; PBL, peripheral blood lymphocyte; HPLC, high-performance liquid chromatography; AZTMP, 3′-azido-2′,3′-dideoxythymidine 5′-monophosphate; d4TTP, 2′,3′-didehydro-2′,3′-dideoxythymidine-5′-triphosphate; ddAMP, 2′,3′-dideoxyadenosine-5′-monophosphate; d4AMP, 2′,3′-didehydro-2′,3′-dideoxyadenosine-5′-monophosphate; FddAMP, 2′-fluoro-ddAMP.

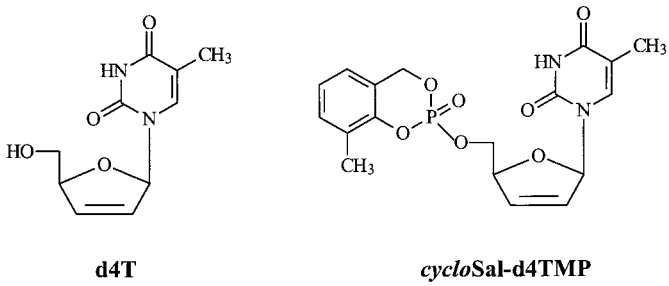
MT-4 cell cultures and has also proved to be antivirally effective against HIV in thymidine kinase-deficient CEM/TK$^-$ cells (Meier et al., 1997a, 1998). Under similar experimental conditions, d4T failed to be antivirally active in CEM/TK$^-$ cells because of its dependence on cytosolic TK for its conversion to the 5′-monophosphate form. The cyclosaligenyl prodrug approach is distinct from other prodrug approaches such as the bis(pivaloyloxymethyl)- (Farquhar et al., 1995), bis(*S*-acyl-2-thioethyl)- (Girardet et al., 1995), and aryloxyphosphoramidate (Balzarini et al., 1996; McGuigan et al., 1996) nucleotide prodrugs in that the cyclosaligenyl prodrugs are designed to selectively and directly deliver nucleotides as 5′-monophosphates by a controlled chemically induced hydrolysis. The release of d4TMP from this prodrug occurs according to a coupled cleavage of the two phosphate ester bonds of a neutral precursor molecule through a concerted tandem mechanism and the nucleotide 5′-monophosphate, and the masking group is released in a 1:1 ratio (Fig. 2). The rationale of this concept is based on the different stability of the phenyl- and the benzyl-ester bonds.

The intracellular metabolism of cycloSal-d4TMP and of the parental d4T drug has now for the first time been studied in a variety of cell lines, including peripheral blood lymphocytes (PBLs), monocyte/macrophages, and thymidine kinase-competent and -deficient CEM cells. This study has revealed striking differences between d4T and cycloSal-d4TMP. Our findings provide a pharmacological explanation for the differential antiviral activities of cycloSal-d4TMP and d4T noted in different cell systems and identified cytosolic 5′(3′)-deoxyribonucleotidase as an important enzyme determining the success of the nucleotide prodrug approach to eventually form the nucleotide 5′-triphosphate metabolite.

Materials and Methods

Cells. CEM, Raji, and Daudi cells were obtained from the American Tissue Culture Collection (Rockville, MD). Molt4/clone 8 cells were provided by N. Yamamoto, Tokyo Medical and Dental University (Tokyo, Japan). The thymidine kinase-deficient CEM/TK$^-$ cells were a kind gift from Prof. S. Eriksson (currently at Uppsala University, Uppsala, Sweden) and Prof. A. Karlsson (Karolinska Institute, Stockholm, Sweden). Human primary macrophages were obtained from blood of HIV-seronegative individuals. Mononuclear cells were separated from blood by a Ficoll gradient and layered onto plastic dishes for 5 to 7 days. At the end of this incubation period, nonadherent cells were carefully removed by repeated washings. Remaining adherent cells were >95% macrophages. Further details about this procedure are described elsewhere (Perno et al., 1996).

Radiochemicals and Compounds. The structural formulae of d4T and cycloSal-d4TMP are depicted in Fig. 1. [*methyl*-^{3}H]d4T (specific radioactivity: 17.4 Ci/mmol) and cycloSal-[*methyl*-^{3}H]d4T (specific radioactivity: 6 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA). Nonradiolabeled d4T was synthesized at the Rega Institute by Prof. P. Herdewijn. Unlabeled cycloSal-d4TMP was synthesized according to a previously published procedure (Meier et al., 1998b). For the synthesis of the "slow"-eluting (R_p) and "fast"-eluting (S_p) cycloSal-[*methyl*-^{3}H]d4TMP diastereomers, [*methyl*-^{3}H]d4T was dissolved in acetonitrile, and 1.8 Eq of 3-methylsalicylchlorophosphane were added at 0°C in the presence of 2 Eq of diisopropylethylamine. After stirring for 30 min, 2 Eq of anhydrous *tert*-butylhydroperoxide as a solution in *n*-decane (5–6 M) were added and stirring was continued for 30 min at room temperature. The crude product was obtained after evaporation of the solvent. Further purification and separation of the R_p and S_p diastereomers was achieved by high-performance liquid chromatography (HPLC) on a Whatman C18 Partisphere 4.6 × 250 mm at a flow of 1 ml/min and a mobile phase of 75% 50 mM KH_2PO_4 plus 5 mM tetrabutylammonium phosphate, and 25% acetonitrile. The retention times of the fast and slow diastereomers were 8.43 and 9.29 min, respectively.



d4T ***cyclo*Sal-d4TMP**

Fig. 1. Structural formulae of d4T (stavudine) and its prodrug cyclosaligenyl-d4TMP (cycloSal-d4TMP).

Metabolism of [*methyl*-^{3}H]d4T and cycloSal[*methyl*-^{3}H]d4TMP. The metabolism of the radiolabeled compounds was studied according to previously established procedures (Balzarini et al., 1991, 1993). Unless explicitly stated otherwise, the slow-eluting R_p diasteromer of cycloSal-d4TMP was used. Wild-type CEM or thymidine kinase-deficient CEM/TK$^-$ cells were seeded at 2 to 4 × 10^5 cells/ml in RPMI-1640 culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. Five-milliliter cell suspensions in 25-cm^2 culture flasks were then incubated with varying concentrations (i.e., 0.1, 1, 10, and 100 μM) of the radiolabeled compounds. At different time intervals (i.e., 2, 6, 24, 48, and 72 h), cells were centrifuged, washed twice with cold RPMI-1640 medium, and precipitated with cold methanol:water (2:1). Adherent macrophages were gently scraped, counted, then centrifuged, and washed as described for the CEM cells. After centrifugation, the supernatants were subjected to HPLC analysis and separated on a Partisphere SAX column (Wattman, Clifton, NJ). A linear gradient of 0.005 M $(NH_4)H_2PO_4$ (pH 5.0) (buffer A) to 0.30 M $(NH_4)H_2PO_4$ (pH 5.0) (buffer B) was used. Equilibration was started for 5 min with buffer A. Then, a linear gradient from 100% buffer A to 100% buffer B was run in 15 min. Buffer B was then kept for 20 min. Next, a linear gradient to 100% buffer A during 5 min was performed, and 100% buffer A was kept for another 5 min before starting the next gradient. The eluate was collected in different fractions and assayed for radioactivity in a toluene-based scintillant.

The metabolism of the R_p cycloSal-d4TMP diastereomer was also compared with the metabolism of the S_p cycloSal-d4TMP diastereomer and with the metabolism of a 1:1 mixture of the R_p plus S_p cycloSal-d4TMP diastereomers. CEM cell cultures were exposed to 0.17 μM the S_p, the *R*p, or the mixture of S_p plus R_p diastereomers, for 24 h, and then d4T metabolites were examined in the cell extracts as described above.

Different human lymphocyte cell lines (i.e., CEM, MT-4, Molt 4/C8, and Daudi cells) and primary cell cultures [i.e., resting and phytohemagglutinin-activated peripheral blood mononuclear cell, and monocyte/macrophages (M/M)] were also exposed to 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP, during 24 h, and thereafter the levels of the metabolites were determined as described above.

In one set of experiments, 0.1 μM [^{3}H]cycloSal-d4TMP was incubated for 24 h with 5 ml of CEM cells (~3 × 10^5 cells/ml) that were suspended in RPMI-1640-based culture medium for which the pH was adjusted to pH 6.9 with H_3PO_4. The pH of normal RPMI-1640-based culture medium is 7.3.

Intracellular Retention of [*methyl*-^{3}H]d4T Metabolites in CEM Cells after Removal of [*methyl*-^{3}H]d4T and cycloSal-[*methyl*-^{3}H]d4TMP from the Cell Culture Supernatant. CEM cells were seeded at 4 × 10^5 cells per ml and incubated with 0.1 μM [*methyl*-^{3}H]d4T or 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP for 24 h. Then, the extracellular drug was removed by centrifugation of the cells and three careful washing of the cell pellet with warm culture

medium. After further incubation of the cells during 0, 2, 4, and 8 h after removal of the radiolabeled compound, cell extracts were prepared and the radiolabeled metabolites determined by HPLC analysis as described above.

Substrate Activity of d4TMP, 3′-azido-2′,3′-dideoxythymidine 5′-phosphate (AZTMP), and dTMP for 5′(3′)-Deoxyribonucleotidase. Purification and characterization of the enzyme was as previously described (Rampazzo et al., 1999). Assays with the recombinant enzyme were made as follows: different amounts of nucleotides were incubated at 37°C for 15 min with the enzyme in 50 mM Tris-maleate, pH 6.0, 20 mM $MgCl_2$, 5 mM dithiothreitol, and 0.2 mg/ml bovine serum albumin. The reaction was terminated with 1.2 M H_2SO_4, and phosphate formation was determined (Geladopoulos et al., 1991). K_m and V_{max} values were estimated by linear regression analysis of Lineweaver-Burk reciprocal plots. One enzyme unit corresponds to the formation of 1 μmol of phosphate/min.

Results

Metabolism of cycloSal-d4TMP and d4T in CEM Cell Cultures in Function of Time. CEM cell cultures were exposed to 0.1 μM [*methyl*-^{3}H]d4T and 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP during different time periods ranging between 2 and 72 h (Table 1). At all time points, the most prominent metabolite was d4TTP, irrespective of the incubation time period. However, there were striking differences in the levels and the kinetics of d4TTP formation in [*methyl*-^{3}H]d4T-, as compared to cycloSal-[*methyl*-^{3}H]d4TMP-exposed cells. In d4T-exposed CEM cells, no striking differences in d4TTP levels were observed throughout the whole incubation period. Indeed, d4TTP levels derived from d4T-exposed cells ranged between 17 and 42 pmol/10^9 cells and peaked at 6 to 24 h. In contrast, the d4TTP levels in cycloSal-d4TMP-exposed cells ranged between 25 and 692 pmol/10^9 cells. The highest d4TTP levels were observed at 6 h and rapidly decreased upon further incubation, an observation that may be due to partial conversion of cycloSal-d4TMP to d4T in the extracellular medium at longer incubation times (Table 1). Thus, in contrast with d4T-exposed cells, the intracellular d4TTP levels rapidly peaked in cycloSal-d4TMP-exposed CEM cells but also decreased relatively rapidly thereafter. The d4TTP levels followed closely the appearance of the d4TMP levels in both d4T- and cycloSal-d4TMP-treated cells and were invariably 2- to 3-fold higher than the d4TMP levels. The d4TDP levels were invariably 5 to 10% of the d4TTP levels (data not shown). There was a slight trend to a decrease in the intracellular d4TTP/d4TMP ratios for the cycloSal-d4TMP-treated CEM cells upon longer incubation times (Fig. 3).

Metabolism of the Slow-Eluting R_p and Fast-Eluting S_p Diastereomer of cycloSal-d4TMP in CEM Cells in Function of Incubation Time. Because cycloSal-d4TMP occurs as two diastereomers, both radiolabeled compounds were separated on an HPLC RP C18 column and evaluated for their metabolic properties. Also, an equal mixture of the diastereomers was made and exposed to the CEM cell cultures side-by-side with the separate diastereomers. The two diastereomers were designated slow-eluting R_p and fast-eluting S_p referring to their elution profile on HPLC. Whereas exposure of cycloSal-d4TMP (R_p) to CEM cells resulted in marked levels of d4TMP (i.e., 234, 91, and 29 pmol/10^9 cells after 6, 24, and 48 h, respectively), the cycloSal-d4TMP (fast-eluting S_p) diastereomer released markedly lower levels of d4TMP into the cells (i.e., 29, 16, and 10 pmol/10^9 cells after 6, 24, and 48 h, respectively). These levels are 8-, 5-, and 3-fold lower than those recorded for the slow-eluting R_p diastereomer after 6, 24, and 48 h of incubation (Table 2). The release of lower d4TMP levels also resulted in the formation of lower d4TDP and d4TTP levels. Again, d4TTP was formed at a 7-, 4-, and 2-fold lower extent in the cells exposed to the fast-eluting S_p diastereomer than to the slow-eluting R_p diastereomer after an incubation period of 6, 24, or 48 h. To assess whether the slow- and fast-eluting diastereomers influenced the d4TMP release from one another, a 1:1 mixture of the slow- and fast-eluting diastereomers was exposed to the CEM cell cultures. The d4TMP and eventual d4TTP levels obtained after 6, 24, and 48 h were clearly intermediate between those that were recorded when the S_p and R_p diastereomers were used singly. Thus, from these data it could be concluded that the S_p diastereomer had no influence on the d4TMP release and metabolism of the R_p diastereomer, and vice versa (Table 2).

Metabolism of cycloSal-d4TMP and d4T in CEM Cell Cultures after 24 h in Function of Initial Drug Concentration. Because a 24-h incubation time period resulted in the appearance of the highest d4T metabolite levels, metabolism of d4T and cycloSal-d4TMP at different initial drug concentrations was evaluated after a 24-h incubation. Formation of d4TMP, d4TDP, and d4TTP increased proportionally with higher input concentrations of d4T (Fig. 4). At an

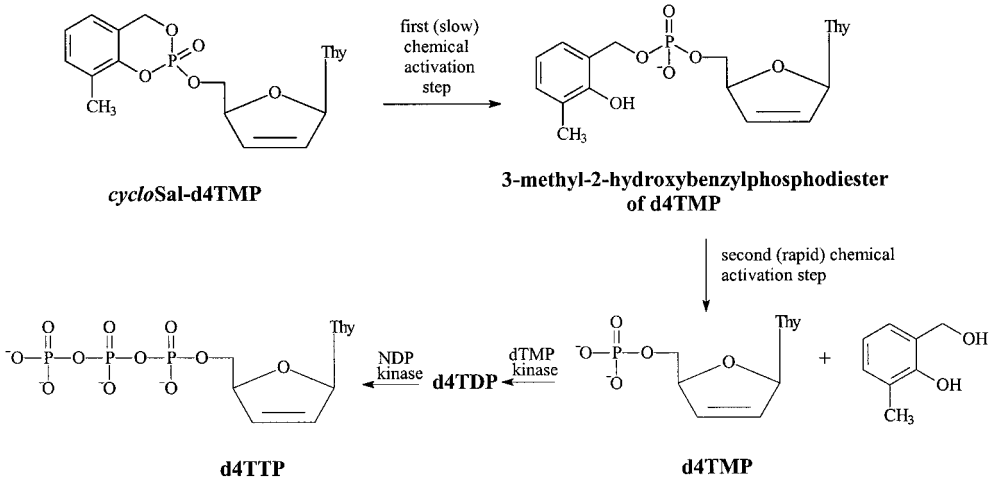


Fig. 2. Selective delivery of d4TMP by a chemically induced tandem reaction from the neutral lipophilic cycloSal-d4TMP prodrug.

initial concentration of 0.1 μM d4T, 32 pmol of d4TTP/10^9 CEM cells appeared intracellularly within 24 h of incubation, and 4- and 30-fold lower concentrations of d4TMP and d4TDP were recorded. A serial (10-fold) increase of the initial extracellular concentration of d4T resulted in a concomitant increase of the intracellular amounts of d4TMP, d4TDP, and d4TTP. The ratios of d4TTP/d4TMP gradually decreased in function of the increasing initial drug concentrations and was equal to 1 at 100 μM d4T (Figs. 4 and 5). When different concentrations of cycloSal-d4TMP were exposed to CEM cell cultures, the d4TMP, d4TDP, and d4TTP levels increased proportionally within the initial concentration range of 0.1 to 10 μM cycloSal-d4TMP. At a 10-fold higher prodrug concentration (i.e., 100 μM), concomitantly (9-fold) increased d4TMP levels were noted, whereas further conversion of d4TMP to d4TDP and d4TTP appeared to level off. In fact, only 2-fold higher d4TDP and less than 1.5-fold higher d4TTP levels were noted upon 100 μM prodrug exposure compared with 10 μM prodrug exposure (Figs. 4 and 5). This observation may suggest that the rates of the conversion of d4TMP to d4TDP and d4TTP may become saturated or that d4TMP has an inhibitory effect on its own further conversion by dTMP kinase, at the highest concentration (100 μM).

TABLE 1

Metabolism of 0.1 μM [*methyl*-^{3}H]d4T and 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP in CEM cell cultures in function of incubation time

Data are the means for two to four independent experiments. The metabolic studies were performed with the slow-eluting R_p diastereomer of cycloSal-d4TMP.

Time	[*methyl*-^{3}H]d4T			
	d4T (2–3)[a]	d4TMP (9–10)[a]	d4TDP (15–16–17)[a]	d4TTP (25–26–27)[a]
h	*pmol / 10^9 cells*			
2	97	16	1.8	17
6	102	22	3.7	42
24	110	13	3.0	37
48	73	10	5.3	33
72	47	6.3	3.0	22
Time	cycloSal-[*methyl*-^{3}H]d4TMP			
	d4T + Prodrug (2–3)[a]	d4TMP (9–10)[a]	d4TDP (16–17)[a]	d4TTP (25–26)[a]
h	*pmol / 10^9 cells*			
2	89	270	19	377
6	95	336	31	692
24	85	93	19	296
48	92	28	9.4	81
72	55	12	3.6	25

[a] Elution times (min) (or fraction numbers) of the metabolites.

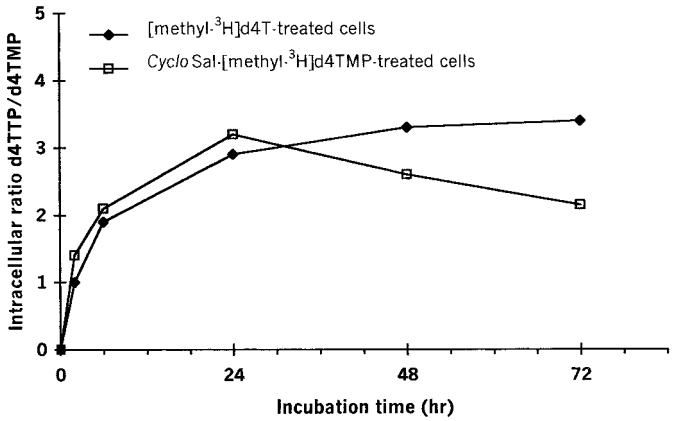


Fig. 3. Intracellular d4TTP/d4TMP ratios in [*methyl*-^{3}H]d4T (♦) and cycloSal-[*methyl*-^{3}H]d4TMP (□)-exposed CEM cells in function of incubation time.

Intracellular Half-Life of d4TMP in d4T- and cycloSal-d4TMP-Treated CEM Cell Cultures. CEM cell cultures were incubated with 0.1 μM [*methyl*-^{3}H]d4T and 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP for a 24-h period, after which the drugs were removed from the extracellular medium by careful washing. At 0, 2, 4, and 8 h after removal of the drugs, intracellular d4T metabolite levels were determined. No striking differences were noted between d4T- and its cycloSal-d4TMP prodrug-treated cells with regard to the decay of d4T metabolites (data not shown). In both cases, the d4TTP levels decreased progressively at a 50% intracellular decay rate of ~3.5 h and reached 15% (d4T exposure) or 14% (cycloSal-d4TMP exposure) of the initial d4TTP values at 8 h after the start of the decay experiments. The progressive decrease of d4TTP levels followed closely the decay of d4TMP (and d4TDP) levels. Statistical analysis revealed that there was no significant difference at 2, 4, or 8 h between the decay of the d4TTP levels in d4T- and cycloSal-d4TMP-treated cells.

Metabolism of [methyl-^{3}H]d4T and cycloSal-[*methyl*-^{3}H]d4TMP in Wild-Type and Thymidine Kinase-Deficient CEM/TK$^-$ Cells. The metabolism of 0.1 μM [*methyl*-^{3}H]d4T and 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP was evaluated comparatively in thymidine kinase-deficient CEM cells and wild-type CEM cells (Tables 1 and 3). Whereas wild-type CEM cells converted d4T to its 5′-mono- plus 5′-di- plus 5′-triphosphate derivatives to the total amount of 35, 68, 53, and 48 pmol/10^9 cells following 2, 6, 24, or 48 h of incubation with d4T (Table 1), the CEM/TK$^-$ cells did not show detectable d4TMP and d4TDP levels (<0.3 pmol/10^9 cells) and only minor d4TTP levels, ranging between 0.66 and 2.0 pmol/10^9 cells within 2 to 24 h (Table 3).

In wild-type CEM cells, cycloSal-d4TMP generated markedly higher levels of d4TMP than did d4T. Thus, total levels of phosphorylated d4T metabolites from cycloSal-d4TMP amounted to 666, 408, and 118 pmol/10^9 cells after 2, 24, or 48 h, respectively (Table 1). In marked contrast with d4T, cycloSal-d4TMP exposure to CEM/TK$^-$ cell cultures resulted in total levels of phosphorylated d4T metabolites that were 152, 282, or 92 pmol/10^9 cells, after 2, 20, or 44 h of incubation, respectively (Table 3). Thus, the total amounts of phosphorylated d4T metabolites, including d4TTP, were quite

TABLE 2

Metabolism of cycloSal-[*methyl*-^{3}H]d4TMP (R_p and S_p diastereomers) in CEM cells in function of time

Data are the means for two independent experiments.

Time	d4T + Prodrug (fr 2–3)[a]	d4T-MP (fr 9–10–11)[a]	d4T-DP (fr 15–16)[a]	d4T-TP (fr 25–26)[a]
h	*pmol / 10^9 cells*			
cycloSal-[*methyl*-^{3}H]d4TMP (R_p diastereomer)				
6	64	234	16	334
24	56	91	7.6	151
48	74	29	7.1	61
cycloSal-[*methyl*-^{3}H]d4TMP (S_p diastereomer)				
6	31	29	1.5	49
24	45	16	1.8	36
48	65	10	2.8	32
cycloSal-[*methyl*-^{3}H]d4TMP (R_p + S_p diastereomers)				
6	46	146	8.2	181
24	79	49	5.1	101
48	68	20	5.5	46

[a] Elution times (min) (or fraction numbers) of the metabolites.

comparable for cycloSal-d4TMP-treated wild-type CEM and CEM/TK$^-$ cells after 44 to 48 h. Whereas d4T was unable to generate pronounced d4TTP levels in TK-deficient CEM cells (~20- to 25-fold lower than in wild-type cells), cycloSal-d4TMP was able to do so at a fairly comparable extent as in wild-type CEM cells (~400-fold higher d4TTP levels in prodrug-exposed CEM/TK$^-$ cells compared with d4T-exposed CEM/TK$^-$ cells; Table 3).

Metabolism of cycloSal-d4TMP in Wild-Type CEM Cells Suspended in Culture Medium at pH 6.9 of 24 h of Incubation. The chemical half-life of cycloSal-d4TMP at pH 7.3 (the pH of normal cell culture medium) is approximately 10 h but is markedly increased at lower pH values (i.e., ~35 h at pH 6.9). Therefore, cycloSal-d4TMP metabolism was investigated in CEM cells that were suspended in culture medium that was adjusted to pH 6.9. No marked differences were observed for the formation of d4TMP, d4TDP, and d4TTP from cycloSal-d4TMP, regardless of the (extracellular) pH of the cell culture medium. At both pH 6.9 and 7.3, d4TMP, d4TDP, and d4TTP levels were formed to a comparable extent (i.e., 115, 12, and 164 pmol/10^6 cells at pH 6.9 versus 93, 19, and 296 pmol/10^6 cells at normal pH).

Metabolism of cycloSal[*methyl*-^{3}H]d4TMP in Cell Types from Different Origin. The metabolism of cycloSal-[*methyl*-^{3}H]d4TMP was investigated in several different cell types, including the T4-lymphocyte CEM, MT-4, and Molt 4/clone 8 cells, the B lymphocyte Daudi cells, activated and resting PBLs, and primary M/M. Release of d4TMP and metabolism to d4TTP from the cycloSal-d4TMP occurred at a comparable extent in the T and B lymphocyte cell lines. The d4TMP levels (after 24 h of incubation) ranged between 41 and 93 pmol/10^9 cells and the d4TTP levels ranged between 207 and 585 pmol/10^9 cells (Table 4). In all cases, the d4TTP levels were 3.5- to 7-fold higher than the d4TMP levels. In contrast, M/M formed lower but significant d4TMP (21 pmol/10^9 cells) and d4TTP (79 pmol/10^9 cells) levels than the lymphocyte cell lines, and activated PBLs converted cycloSal-d4TMP to an even lower extent than M/M. The d4T metabolite levels in activated PBLs derived from cycloSal-d4TMP (Table 4) were comparable to those derived from d4T in activated PBLs (4.2, 3.1, 0.47, and 13 pmol/10^9 cells for d4T, d4TMP, d4TDP, and d4TTP, respectively). cycloSal-d4TMP exposed to resting PBLs generated d4TMP and d4TTP levels above detection limit (Table 4), whereas d4T metabolites were under the detection limit when d4T was given instead of cycloSal-d4TMP to resting PBL. The d4T plus prodrug levels in (activated) PBLs and M/M are lower than those obtained for the laboratory cell lines (Table 4). However, it should be noted that the experimental procedure to measure the phosphorylated d4T metabolites is not suitable to determine the intracellular d4T plus prodrug levels in an accurate way. Therefore, it is unclear whether the lower d4T plus prodrug levels observed in PBLs and M/M represent a real difference with those observed in the laboratory cell lines.

Substrate Efficiency of dTMP, d4TMP, or AZTMP for 5′(3′)-Deoxyribonucleotidase. Among the known 5′-nucleotidases, the recently cloned human 5′(3′)-deoxyribonucleotidase (Rampazzo et al., 1999) appeared to be the best potential candidate for the intracellular hydrolysis of the phosphorylated prodrugs. This enzyme prefers deoxyribonucleotides over ribonucleotides, with dUMP and dTMP giving

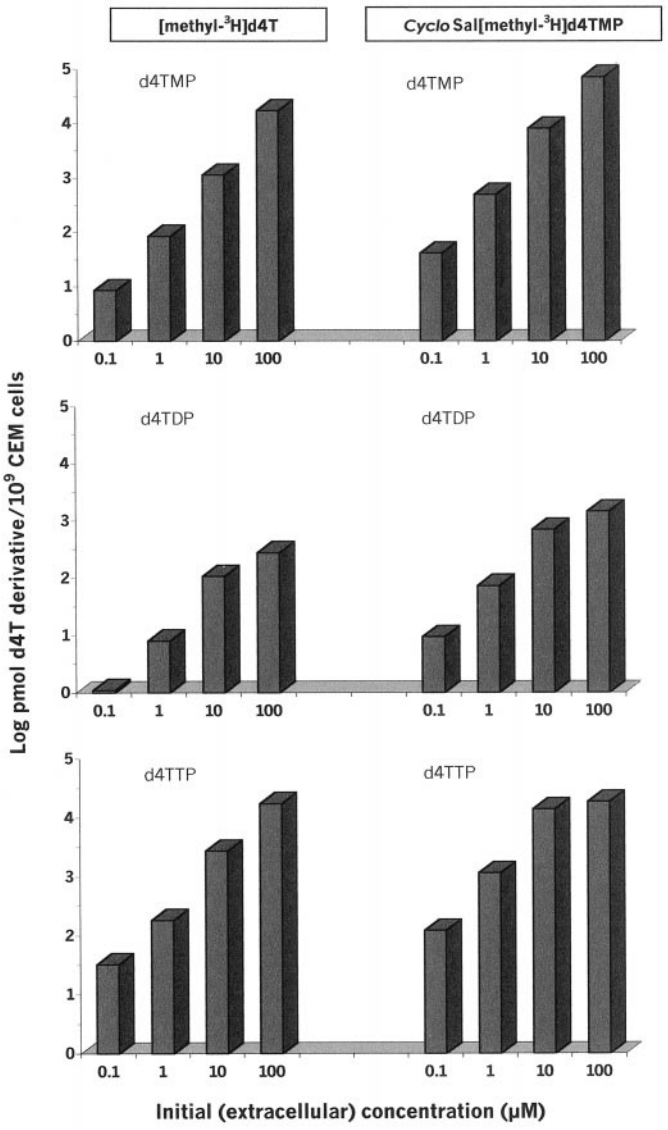


Fig. 4. Intracellular levels of [*methyl*-^{3}H]d4TMP, [*methyl*-^{3}H]d4TDP, and [*methyl*-^{3}H]d4TTP following incubation of CEM cells for 24 h at different initial concentrations of [*methyl*-^{3}H]d4T and cycloSal-[*methyl*-^{3}H]d4TMP.

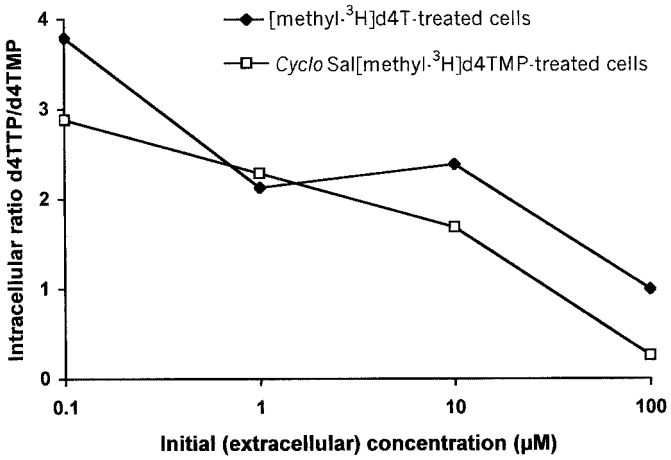


Fig. 5. Intracellular d4TTP/d4TMP ratio in [*methyl*-^{3}H]d4T (♦) and cycloSal-[*methyl*-^{3}H]d4TMP (□)-exposed CEM cells in function of initial drug concentration.

the best activity. In a comparison of the dephosphorylation of the natural substrate dTMP with the two phosphorylated prodrugs, d4TMP and 3AZTMP we found that the substrate efficiency (V_{max}/K_m) of d4TMP was less than one-tenth of dTMP, whereas AZTMP was a slightly better substrate than dTMP. This suggests that in intact cells d4TMP remains available for a much longer time than AZTMP and therefore can be more efficiently phosphorylated to the metabolically active triphosphate.

Discussion

We have investigated the metabolic fate of the cyclosaligenyl prodrug of d4T under a variety of experimental conditions. The cyclosaligenyl prodrug approach is distinct from the other prodrug approaches because the cyclosaligenyl prodrug is the only known prodrug approach for which the nucleotide release is chemically driven. It has been shown by Meier et al. (1997a,b, 1998) that the chemical half-life can be markedly influenced by the introduction of electron-withdrawing or electron-donating substituents (resulting in chemical half-lives of less than 1 h to more than 20 h). Also, the pH has a marked influence on the half-life of the cyclosaligenyl prodrugs. In this study, we investigated the metabolism of the 3-methyl cycloSal-d4TMP derivative. The 3-methyl derivative was previously found to be endowed with the most pronounced antiviral potency, not only with d4TMP as the coupled nucleotide but also with other nucleotides such as AZTMP, ddAMP, d4AMP, and FddAMP (Meier et al., 1997a,b, 1998, 1999a,b).

The cyclosaligenyl prodrugs consist of two diastereomers that are eluting at a close distance to one another on HPLC. It was interesting to note that the slow-eluting (R_p) diastereomer released markedly more d4TMP and (thus) also resulted in higher intracellular d4TTP levels than its fast-eluting (S_p) diastereomer. We have previously found that the R_p diastereomers of a variety of cycloSal-d4TMP prodrugs containing different substituents at the 3 and/or 5 position of the phenyl ring (i.e., 3-methoxy, 3-methyl, 3,5-dimethyl) invariably showed a higher antiviral activity than their S_p congeners (Meier et al., 1998b). These antiviral (HIV) data are in full agreement with the different metabolic behaviors of both R_p and S_p diastereomers of the cycloSal-d4TMP prodrug examined in this study.

Our metabolic data are also in full agreement with the antiviral activity of the cycloSal-d4TMP prodrug in wild-type and TK-deficient CEM/TK$^-$ cells. Indeed, comparable d4TTP levels have been found in prodrug-treated CEM and CEM/TK$^-$ cells, and the antiviral potency of cycloSal-d4TMP has also been found to be comparable in both cell lines (IC_{50} for HIV-2: 0.12 and 0.09 μM in wild-type CEM and CEM/TK$^-$ cells, respectively). These observations are in sharp contrast with the low antiviral activity of the parent d4T compound (and the corresponding low d4TTP levels shown in this study) in CEM/TK$^-$ cells (Meier et al., 1998). Similar observations in CEM/0 and CEM/TK$^-$ cells were made earlier for the aryloxyalaninyl d4TMP phosphoramidate prodrug So324, which releases d4TMP by an enzymatically driven reaction mechanism (Balzarini et al., 1996; Saboulard et al., 1999). Also, it should be mentioned that the intracellular d4TTP levels, formed from So324 and from cycloSal-d4TMP, are very similar in CEM cells at comparable extracellular drug concentrations and incubation times (i.e., 1 μM, 24 h; Balzarini et al., 1996). The independence of the activation of the d4TMP prodrugs on cytosolic thymidine kinase may be of considerable clinical importance, because two separate groups (Jacobsson et al., 1995; Antonelli et al., 1996) have shown that resistance to azidothymidine treatment of HIV infection may be related to loss of thymidine kinase activity in the patients' peripheral blood lymphocytes.

In contrast to cycloSal-d4TMP, cycloSal-AZTMP fails to show pronounced activity in CEM/TK$^-$ cells (Meier et al., 1997b). Our metabolic studies with radiolabeled cycloSal-AZTMP revealed, indeed, very low levels of AZTMP, AZTDP, and AZTTP in cycloSal-AZTMP-exposed CEM/TK$^-$ cells (in contrast with wild-type CEM cells; Balzarini et al., 1999). However, we have also found that in extracts from CEM cells AZTMP is more rapidly dephosphorylated than d4TMP (21% conversion of 500 μM AZTMP to AZT within 6 h, versus 1.8% conversion of 500 μM d4TMP to d4T). This prompted a search for the enzyme responsible for the differential stability of the two nucleotides. A likely candidate was 5′(3′)-deoxynucleotidase (Fritzson and Smith, 1971; Höglund and Reichard, 1990; Rampazzo et al., 1999), the only known cytosolic nucleotidase to show a preference for deoxynucleoside monophosphates as substrates. We now find that 5′(3′)-deoxyribonucleotidase indeed fulfills the conditions for the enzyme we were looking for. The differences in V_{max}/K_m values (Table 5) indicate that the recombinant enzyme hydrolyzes AZTMP approximately 20 times faster than d4TMP. This is the first demonstration of the importance of a 5′-nucleotidase concerning the antiviral efficacy of nucleotide analogs. It is possible that nucleoside analogs with a low affinity for the phosphorylating kinase may turn out to be effective as pro-

TABLE 3

Metabolism of 0.1 μM [*methyl*-^{3}H]d4T and 0.1 μM cycloSal-[*methyl*-^{3}H]d4TMP in CEM/TK$^-$ cell cultures in function of incubation time

Data are the means for two independent experiments. The metabolic studies were performed with the slow-eluting R_p diastereomer of cycloSal-d4TMP.

Time	d4T + Prodrug (fr 2–3)[a]	d4T-MP (fr 10–11)[a]	d4T-DP (fr 15–16–17)[a]	d4T-TP (fr 24–25–26)[a]
h		*pmol / 10^9 cells*		
cycloSal-[*methyl*-^{3}H]d4TMP				
2	40	35	6.5	111
20	55	32	14	236
44	35	8.8	6.5	77
[*methyl*-^{3}H]d4T				
2	32	<0.3	<0.3	0.66
6	12	<0.3	<0.3	2.0
24	8.2	<0.3	<0.3	1.5

[a] Elution times (min) (or fraction numbers) of the metabolites.

drugs if their monophosphates are poor substrates for nucleotidases. Thus, in the case of cycloSal-d4TMP and cycloSal-AZTMP, d4TMP released from cycloSal-d4TMP may be predominantly anabolized to d4TTP rather than catabolized to d4T by the hydrolytic enzyme(s), whereas AZTMP released from cycloSal-AZTMP may be predominantly catabolized to AZT by the hydrolytic enzyme(s), rather than further anabolized to AZTTP by the thymidylate kinase, which is known to poorly convert AZTMP to AZTDP (Furman et al., 1986).

No marked differences were found in the intracellular appearance of d4TMP from cycloSal-d4TMP within 24 h when the prodrug was exposed to the CEM cell cultures at different pH values (pH 6.9 or 7.3). These observations indicate that the major amount of conversion to d4TMP from the prodrug occurs intracellularly where no pH differences are expected to occur under both experimental conditions. Also, it is not fully clear whether enzymatic conversion, in addition to chemical conversion, of cycloSal-d4TMP prodrug may play a role in the eventual intracellular formation of d4TMP. Meier et al. (1998) have recently shown that the hydrolysis half-life of a variety of cyclosaligenyl derivatives could be shortened when exposed to the cell culture medium in the presence of fetal calf serum (10%) compared with the same cell culture medium in the absence of fetal calf serum. These observations may point to an enzymatic contribution to the conversion of the cyclosaligenyl prodrugs. If this would be the case, the hydrolysis of the prodrug and subsequent formation of d4TTP will turn out to be a complex interplay of chemical and enzymatic processes with different pharmacological kinetics. However, other studies have also shown that freshly prepared CEM and mouse liver extracts did not markedly influence the hydrolysis rate of the cyclosaligenyl prodrugs when exposed to the prodrugs as such or after being heated for 30 min at 95°C (data not shown). This issue is now under further investigation in our laboratories.

TABLE 4

Metabolites of 1 μM cycloSal-[*methyl*-^{3}H]d4TMP in CEM, MT-4, Molt 4/C8, DAUDI, PBL, and M/M cells after 24 h of incubation

Data are the means for two independent experiments (except for the data obtained in CEM cell cultures, which were the means for four independent experiments). The metabolic studies were performed with the slow-eluting R_p diastereomer of cycloSal-d4TMP.

Cell Line	d4T + Prodrug (fr 2–3)[a]	d4T-MP (fr 9–10–11)[a]	d4T-DP (fr 15–16)[a]	d4T-TP (fr 25–26)[a]
		pmol / 10^9 cells		
CEM	85	93	19	296
MT-4	37	41	17	207
MOLT 4/C8	60	74	24	472
DAUDI	40	78	19	585
PBL	2.7	1.1	ND[b]	0.2
PBL (activated)	16	8.8	0.2	5.9
M/M	15	21	8.8	79

[a] Elution times (min) (or fraction numbers) of the metabolites.
[b] ND, not detectable.

TABLE 5

Substrate efficiency of dTMP, d4TMP, or AZTMP for 5′ (3′)-deoxyribonucleotidase

Substrate	K_m	V_{max}	Substrate Efficiency
	mM	*units*[a] */ mg*	V_{max}/K_m
dTMP	1.25	347	277
AZTMP	0.41	187	456
d4TMP	1.47	33	22

[a] Units = μmol/min.

In conclusion, we have investigated the intracellular metabolism of a novel prodrug of d4T, cycloSal-d4TMP, and demonstrated the pharmacological basis of the antiviral behavior of this prodrug in several cell systems. Our data have indicated that d4TMP is released intracellularly from cycloSal-d4TMP in a variety of cell systems, including thymidine kinase-deficient cells. They have also revealed the stability of d4TMP against the hydrolytic activity of 5′(3′)deoxynucleotidase, thus providing a dual basis for the efficient anabolic pathway of cycloSal-d4TMP to d4TTP, that is through an increased delivery of d4TMP coupled with an increased resistance of the latter to enzymatic breakdown.

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