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Intracellular Metabolism of *Cyclo*Saligenyl 3′-Azido-2′,3′-dideoxythymidine Monophosphate, a Prodrug of 3′-Azido-2′,3′-dideoxythymidine (Zidovudine)

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ABSTRACT

The administration of *Cyclo*Saligenyl 3′-azido-2′,3′-dideoxythymidine monophosphate (*Cyclo*Sal-AZTMP) to CEM cells resulted in a concentration- and time-dependent conversion to the 5′-monophosphate (AZTMP), 5′-diphosphate (AZTDP), and 5′-triphosphate (AZTTP) derivatives. High ratios of AZTMP/AZTTP were found in the CEM cell cultures treated with *Cyclo*Sal-AZTMP. The intracellular $T_{1/2}$ of AZTTP in CEM cell cultures treated with either AZT and *Cyclo*Sal-AZTMP was approximately 3 h. A variety of human T- and B-lymphocyte cell lines efficiently converted the prodrug to the AZT metabolites, whereas peripheral blood lymphocytes and primary monocyte/macrophages showed at least 10-fold lower metabolic conversion of the prodrug. *Cyclo*Sal-AZTMP failed to generate marked levels of AZT metabolites in thymidine kinase-deficient CEM/TK$^-$ cells, an observation that is in agreement with the substantial loss of antiviral activity of *Cyclo*Sal-AZTMP in CEM/TK$^-$ cells. The inability of *Cyclo*Sal-AZTMP to generate AZTMP in CEM/TK$^-$ cells is presumably due to a relatively high hydrolysis rate of AZTMP to the parent nucleoside AZT, combined with the inability of CEM/TK$^-$ cells to phosphorylate AZT to AZTMP through the cytosolic salvage enzyme thymidine kinase.

Many antiviral nucleoside analogs possess a poor affinity for their activating (phosphorylating) enzyme [i.e., 2′,3′-dideoxycytidine (ddC; zalcitabine) for deoxycytidine kinase, 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T; stavudine) for thymidine kinase (TK), and 2′,3′-dideoxyinosine (ddI; didanosine) for 5′-nucleotidase] (Furman et al., 1986; Balzarini et al., 1987a,b; Starnes and Cheng, 1987; Johnson and Fridland, 1989). Therefore, several attempts have been undertaken to design nucleoside monophosphate prodrugs that directly deliver the 5′-monophosphate derivative of nucleoside analogs (i.e., 2′,3′-dideoxynucleosides) into the intact target cells. To date, at least four different approaches have been followed to achieve this goal: 1) bis pivaloyloxymethyl (POM) derivatives (Farquhar et al., 1994, 1995; Pompon et al; 1994), 2) bis(S-acyl-2-thioethyl) (SATE) derivatives (Girardet et al., 1995; Valette et al., 1996), 3) aryloxyphosphoramidate (APA) derivatives (Balzarini et al., 1996; McGuigan et al., 1996a,b), and 4) *Cyclo*Sal derivatives (Meier et al., 1997a,b, 1998a,b; for an overview, see Meier, 1998). When applied to some thymidine monophosphate analogs, all four types of prodrugs have been shown to retain biological activity in TK-deficient cells, pointing to an efficient delivery of the nucleoside 5′-monophosphate inside the intact cells. However, the success and efficiency of the nucleoside kinase-bypass prodrug approach may also depend on the nature of the nucleoside analog. For example, the APA and *Cyclo*Sal prodrug of d4TMP proved highly successful in retaining antiviral activity in human immunodeficiency virus (HIV)-infected TK$^-$ cells, whereas the parent compound d4T had no antiviral activity in this cell line (Balzarini et al., 1996; Meier et al., 1997a). Studies with radiolabeled d4T and the APA prodrug of d4T revealed a virtual lack of phosphorylation of d4T in CEM/TK$^-$ cells, whereas the prodrug of d4TMP was able to produce considerable levels of d4TMP, d4TDP, and d4TTP in CEM/TK$^-$ cells (Balzarini et al., 1996). However, when the same prodrug approach was applied on AZTMP, the APA and *Cyclo*Sal prodrugs of AZTMP had no marked anti-HIV activity in CEM/TK$^-$ cells (McGuigan et al., 1996b;

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ABBREVIATIONS: *Cyclo*Sal-AZTMP, *Cyclo*Saligenyl 3′-azido-2′,3′-dideoxythymidine monophosphate; AZTMP, 5′-monophosphate derivative of AZT; AZTDP, 5′-diphosphate derivative of AZT; AZTTP, 5′-triphosphate derivative of AZT; TK, thymidine kinase; APA, aryloxyphosphoramidate; 5′-Nu, 5′-nucleotidase; PBL, peripheral blood lymphocyte; HIV, human immunodeficiency virus; dTMP, thymidylate; AZT, 3′-azido-2′,3′-dideoxythymidine; M/M, monocyte/macrophages.

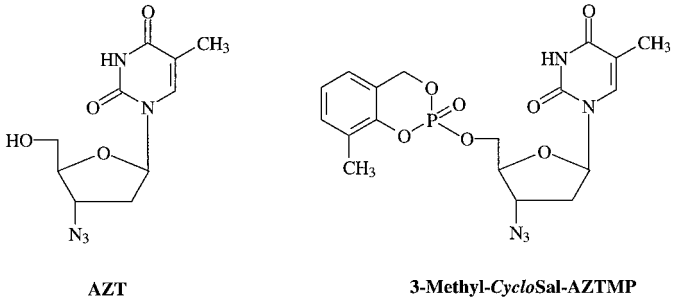
Meier et al., 1997b). Also, the parent AZT was completely devoid of antiviral activity in this cell line. These data suggest that the APA and *Cyclo*Sal prodrugs of AZTMP must have been unable to eventually generate substantial levels of AZTTP to inhibit HIV replication in the CEM/TK$^-$ cells. Thus, although the d4TMP prodrugs proved antivirally effective in HIV-infected CEM/TK$^-$ cells, the AZTMP prodrugs again failed to retain a substantial antiviral potency in CEM/TK$^-$ cells. It is unclear, however, why the TK bypass concept succeeds with APA and *Cyclo*Sal prodrugs of d4TMP but not with the corresponding prodrugs of AZTMP. In an attempt to clarify this issue, *Cyclo*Sal-AZTMP, radiolabeled at the 5-methyl group of AZT, has been synthesized and used to compare the metabolism of AZT and *Cyclo*Sal-AZTMP in a variety of cell lines, including the TK$^-$ CEM/TK$^-$ cells.

Materials and Methods

Cells. CEM, Raji, and Daudi cells were obtained from American Tissue Culture Collection (Rockville, MD). Molt4/clone 8 cells were provided by N. Yamamoto (Yamaguchi University, Yamaguchi, Japan). The TK-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 the blood of HIV-seronegative individuals. Mononuclear cells were separated from blood by Ficoll gradient and layered onto plastic dishes for 5 to 7 days. At the end of this period, nonadherent cells were carefully removed through 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 formulas of AZT and *Cyclo*Sal-AZTMP are depicted in Fig. 1. [CH_3-^{3}H]3′-Azido-2′,3′-dideoxythymidine (AZT; specific radioactivity, 10.9 Ci/mmol) and *Cyclo*Sal[CH_3-^{3}H]AZTMP (specific radioactivity, 14 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA). Nonlabeled AZT was derived from Sigma Chemical Co. (St. Louis, MI). Unlabeled *Cyclo*Sal-AZTMP was synthesized according to a previously published procedure (Meier et al., 1997b). For the synthesis of *Cyclo*Sal[CH_3-^{3}H]AZTMP, 5-[CH_3-^{3}H]AZT was dissolved in acetonitrile, and 1.8 equivalents of 3-methylsalicylchlorophosphane was added at 0°C in the presence of 2 equivalents of diisopropylethylamine. After stirring for 30 min, 2 equivalents of anhydrous *tert*-butylhydroperoxide as a solution in *n*-decane (5–6 M) was added, and stirring was continued for 30 min at room temperature. The crude product was obtained after evaporation of the solvent. Further purification was achieved by HPLC (Whatman C18 Partisphere, 4.6 × 250 mm; flow, 1 ml/min; mobile phase, 80% 50 mM KH_2PO_4, pH 5.5, and 20% acetonitrile).

Metabolism of [CH_3-^{3}H]AZT and *Cyclo*Sal[CH_3-^{3}H]AZTMP. The metabolism of the radiolabeled compounds was studied according to previously established procedures (Balzarini et al., 1991, 1993). Briefly, wild-type CEM or TK-deficient CEM/TK$^-$ cells were seeded at 2 to 4 × 10^5 cells/ml in RPMI 1640 culture medium supplemented with 10% FCS, 2 mM L-glutamine, and 0.075% $NaHCO_3$. The 5-ml cell suspensions in 25-cm^2 culture flasks were then incubated with varying concentrations (i.e., 0.07 or 0.09, 1, 10, and 100 μM; see Table 2) of the radiolabeled compounds. At different time intervals (i.e., 2, 6, 24, 48, and 72 h; see Table 1), cells were centrifuged, washed (twice with cold RPMI 1640 medium), and precipitated with cold methanol/water (2:1). Adherent macrophages were gently scraped, counted, and then centrifuged and washed as described for the CEM cells. After centrifugation, the supernatants were subjected to HPLC analysis. A linear gradient of 0.007 M $(NH_4)H_2PO_4$ (pH 3.8) to 0.25 M $(NH_4)H_2PO_4$ plus 0.5 M KCl (pH 4.5) was used. The eluate was collected in different fractions and assayed for radioactivity in a toluene-based scintillant.



AZT 3-Methyl-*Cyclo*Sal-AZTMP

Fig. 1. Structural formulas of AZT and 3-methyl-*Cyclo*Sal-AZTMP.

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 cells and monocyte/macrophages (M/M)] were also exposed to 0.09 μM [^{3}H]*Cyclo*Sal-AZTMP, during 24 h, after which the levels of the metabolites were determined as described above.

In one set of experiments, 0.07 μM [^{3}H]*Cyclo*Sal-AZTMP (5 μCi) was incubated for 24 h with 5 ml of CEM cell cultures (~3 × 10^5 cells/ml) that were suspended in RPMI 1640-based culture medium for which the pH was adjusted to 6.9 with H_3PO_4. The pH of normal RPMI 1640-based culture medium is 7.32.

Intracellular Retention of [^{3}H]AZT and Its Metabolites in CEM Cells after Removal of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP from Cell Culture Supernatant. CEM cells were seeded at 4 × 10^5 cells/ml and incubated with 0.09 μM [^{3}H]AZT or 0.07 μM [^{3}H]*Cyclo*Sal-AZTMP (5 μCi/5 ml cell culture) for 24 h. Then, the extracellular drug was removed by centrifugation of the cells and three times 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 were determined by HPLC analysis as described earlier.

Results

Phosphorylation of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP in CEM cells as a function of different incubation times. After

TABLE 1
Metabolism of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP in CEM cell cultures in function of incubation time
Values are the mean of two to four independent experiments.

Time	[CH_3-^{3}H]AZT (0.09 μM)			
	AZT (fr 2-3)[a]	AZTMP (fr 8-9-10)[a]	AZTDP (fr 14-15-16)[a]	AZTTP (fr 22-23-24)[a]
h	*pmol/10^9 cells*			
2	80	10,990	313	816
6	112	16,370	412	1,032
24	84	9,473	427	843
48	62	6,868	391	702
72	40	4,187	304	455
	[CH_3-^{3}H]*Cyclo*Sal-AZTMP (0.07 μM)			
	AZT + Prodrug (fr 2-3)[a]	AZTMP (fr 9-10-11)[a]	AZTDP (fr 14-15-16)[a]	AZTTP (fr 22-23-24)[a]
h	*pmol/10^9 cells*			
2	12	171	13	11
6	13	1,215	57	146
24	39	5,475	236	425
48	43	4,786	219	376
72	29	3,304	203	260

[a] The values within parentheses represent the elution times (min) (or fraction numbers) of the radiolabeled metabolites. Each fraction contains 1 ml, collected in 1 min.

the incubation of CEM cells with 0.09 μM [^{3}H]AZT or 0.07 μM [^{3}H]*Cyclo*Sal-AZTMP, the intracellular levels of the parent compounds and their metabolites were measured at different time points during incubation (Table 1). As previously observed, the exposure of CEM cells to AZT resulted in a substantial accumulation of AZTMP at all time points measured, at concentrations that exceeded the intracellular AZTTP levels by 10- to 16-fold. The AZTMP and AZTTP levels were highest after 6-h incubation, after which they progressively decreased. At 72 h, 25% of the highest AZTMP concentration (observed at 6 h) and 40% of the highest AZTTP concentration (observed at 6 h) was still present. The appearance and disappearance of AZTDP closely followed AZTMP and AZTTP levels; the AZTDP levels were ~50% of the AZTTP levels over all time points examined.

When *Cyclo*Sal-AZTMP was exposed to the CEM cell cultures, the formation of AZTMP, AZT-DP, and AZTTP was inferior to that recorded for the parent compound AZT and peaked somewhat later (~24 h) than with AZT (~6 h; Table 1). Strikingly, during the first 2 and 6 h of *Cyclo*Sal-AZTMP exposure, relatively small amounts of AZTMP, AZTDP, and AZTTP were formed relative to the cultures exposed to AZT (<2% after 2 h and ~15% after 6 h). However, after 24, 48, and 72 h of incubation with the AZTMP prodrug, the AZT metabolites were half the amounts of those that were formed when parental AZT was given.

Intracellular Retention of [^{3}H]AZT Metabolites on Removal of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP from Culture Medium. CEM cells were incubated with 0.09 μM [^{3}H]AZT or 0.07 μM [^{3}H]*Cyclo*Sal-AZTMP for a 24-h period, on which the compound was removed from the extracellular medium by thorough washing of the cells (Fig. 2). The decay in phosphorylated AZT metabolites was very similar for the AZT- and *Cyclo*Sal-AZTMP-exposed cell cultures. At 2 h after removal of the radiolabeled compounds from the extracellular medium, the intracellular levels of phosphorylated AZT metabolites had decreased to 40% of the initial value and further progressively decreased to 15% after 8 h. When considering the intracellular decay of AZTTP, the disappearance of this metabolite was somewhat slower than the total amount of metabolites, with levels being 60, 30, and 20% of the initial values after 2, 4, and 8 h, respectively (Fig. 2). The intracellular $T_{1/2}$ of AZTTP was calculated as ~3 h, in both AZT- and *Cyclo*Sal-AZTMP-exposed CEM cell cultures. The intracellular $T_{1/2}$ found in this study is in agreement with the $T_{1/2}$ of AZTTP reported by Furman et al. (1986) and Törnevik et al. (1992) in CEM and other cell lines.

Metabolism of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP in TK-Deficient CEM/TK$^-$ Cell Cultures. Conversion of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP to AZTMP, AZTDP, and AZTTP was examined in the wild-type CEM cell line and its cytosolic TK-deficient counterpart CEM/TK$^-$ (Fig. 3). AZT phosphorylation was dramatically reduced in the AZT-exposed TK-deficient CEM/TK$^-$ cells compared with wild-type CEM cells. In the CEM/TK$^-$ cells, AZTMP levels were below detection limit after 2 h and were four orders of magnitude lower than in wild-type CEM cells after 6 and 24 h. Although no AZTDP could be detected in CEM/TK$^-$ cells, AZTTP was present at levels that were three to four orders of magnitude lower than in wild-type CEM cells (Fig. 3).

Compared with AZT, *Cyclo*Sal-AZTMP was converted to AZTMP and AZTTP at a significantly higher efficiency in the CEM/TK$^-$ cells but still to a much lower extent than in wild-type CEM cells (Fig. 3). After 2 h, the amount of AZTTP formed in CEM/TK$^-$ cells was ~50% compared with wild-type CEM cells. However, the AZTTP levels observed in CEM/TK$^-$ cells after 20 and 44 to 48 h were only 0.1 to 0.3% of the values in wild-type CEM cells. These data suggest that the predominant metabolite of *Cyclo*Sal-AZTMP in CEM/TK$^-$ cells on prolonged exposure is AZT. Interestingly, although the AZTTP levels generated in AZT-exposed CEM/TK$^-$ cells progressively increased as a function of incubation time (i.e., 0.1, 0.3, and 1.1 pmol/10^9 cells after 2, 6, and 24 h, respectively), the AZTTP levels formed from *Cyclo*Sal-AZTMP were highest at 2 h (4 pmol/10^9 cells) and progressively decreased to 1.4 and 0.3 pmol/10^9 cells after 20 and 44 h. Thus, the AZTTP levels measured at 20 to 24 h were similar in CEM/TK$^-$ cells incubated with either AZT or *Cyclo*Sal-AZTMP, yet after 2 h, the AZTTP levels derived from

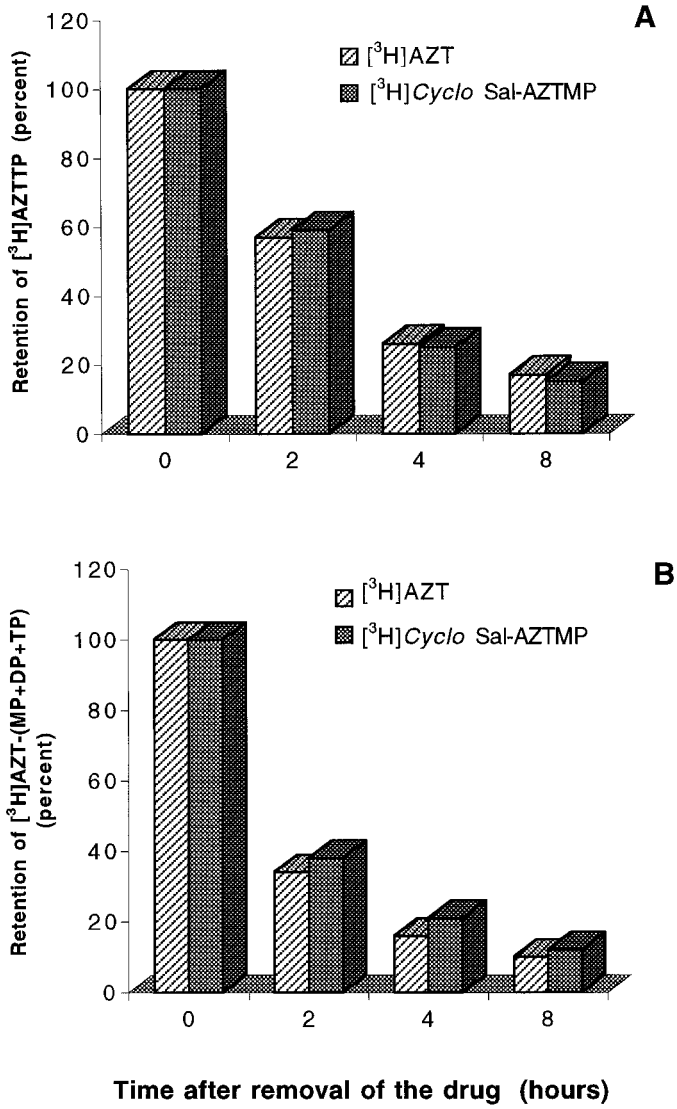


Fig. 2. Decay of intracellular [^{3}H]AZTTP (A) and total [^{3}H]AZTMP plus [^{3}H]AZTDP plus [^{3}H]AZTTP metabolites (B) as a function of time after removal of [^{3}H]AZT or [^{3}H]*Cyclo*Sal-AZTMP from the extracellular medium. The CEM cell cultures were loaded for 24 h with 0.09 μM [^{3}H]AZT or 0.07 μM [^{3}H]*Cyclo*Sal-AZTMP before drug removal from the culture medium.

*Cyclo*Sal-AZTMP were 10-fold higher compared with the AZTTP levels formed in CEM/TK$^-$ cells exposed to AZT.

Metabolism of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP in CEM Cells as a Function of Different Input Concentrations. The formation of AZT metabolites from AZT and *Cyclo*Sal-AZTMP increased progressively with higher input concentrations (Table 2), and no differences were observed in the intracellular levels of phosphorylated AZT metabolites derived from AZT- and *Cyclo*Sal-AZTMP in CEM cell cultures. Although the AZTMP levels markedly increased (up to 50-fold) on increasing the initial AZT or *Cyclo*Sal-AZTMP concentrations from ~0.1 to 100 μM, the AZTTP levels differed by only 5- to 7-fold within an input concentration ranging from 0.1 to 100 μM (Table 2). The markedly slower increase in AZTTP than in AZTMP levels on exposure of increasing extracellular AZT concentrations is in agreement with previously published data in other cell lines (i.e., MT-4; Balzarini et al., 1989). This phenomenon is due to the progressive inhibition of thymidylate (dTMP) kinase on intracellular accumulation of high AZTMP levels (Frick et al., 1988; Balzarini et al., 1989).

Metabolism of [^{3}H]*Cyclo*Sal-AZTMP in Different Cell Lines. Next, we investigated the metabolism of 0.07 μM [^{3}H]*Cyclo*Sal-AZTMP in human T-lymphocyte CEM, MT-4, and Molt4/C8 cells, human B-lymphoblast DAUDI cells, primary (phytohemagglutinin-stimulated and nonstimulated) human peripheral blood lymphocytes (PBLs), and primary M/M (Table 3). The metabolism of AZT and *Cyclo*Sal-AZTMP in resting and activated PBLs and in M/M was included in this comparative study because the metabolic properties of the drugs in these cell lines probably mimic closer the in vivo (patient) situation than the findings in the established laboratory cell lines. AZTTP levels formed in the three T-lymphocyte cell lines after 24-h incubation ranged between 385 and 755 pmol/10^9 cells. AZTMP levels were 5- to 13-fold higher (2002–7854 pmol/10^{9}cells) than the AZTTP levels in these cell lines. The B-lymphoblast DAUDI cells generated the highest AZTMP and AZTTP levels after 24 h, amounting to 11,572 and 1,920 pmol/10^9 cells, respectively. In sharp contrast, activated PBLs and primary M/M produced AZTMP levels of 843 to 1044 pmol/10^9 cells but only 29 to 33 pmol/10^9 cells AZTTP after 24 h. Thus, there was at least one order of magnitude less AZTTP produced in PBLs and M/M than in the T-lymphocyte cell lines. In resting (nonactivated) lymphocytes, AZTMP and AZTTP levels were marginal (2.6 and 0.5 pmol/10^9 cells, respectively).

The levels of AZTMP, AZTDP, and AZTTP in activated and resting PBLs exposed to *Cyclo*Sal-AZTMP were comparable to those derived from the parent drug AZT. Again, activated PBLs produced markedly more phosphorylated AZT metabolites than resting (nonactivated) PBLs (Table 4).

Metabolism of [^{3}H]*Cyclo*Sal-AZTMP in CEM Cells that Were Incubated in Culture Medium at pH 6.9. Because *Cyclo*Sal-AZTMP is much more stable at pH values lower than 7.0 (Meier et al., 1998), metabolism of [^{3}H]*Cyclo*Sal-AZTMP was determined after 24-h drug exposure in CEM cell cultures that were incubated in culture medium for which the pH was adjusted to pH 6.9. The AZTMP, AZTDP, and AZTTP metabolites amounted to 4791, 197, and 357 pmol/10^9 cells, and these levels were comparable to those observed in CEM cell cultures that were incubated in regular culture medium (pH 7.32).

Stability of AZTMP and d4TMP in CEM Cell Extracts. AZTMP and d4TMP have been added to freshly prepared CEM cell extracts at 500 μM. After 6 h of incubation at 37°C, 21% of AZTMP was converted to AZT, whereas only 1.8% of d4TMP was converted to d4T. The higher stability of d4TMP versus AZTMP in CEM cell extracts found in this

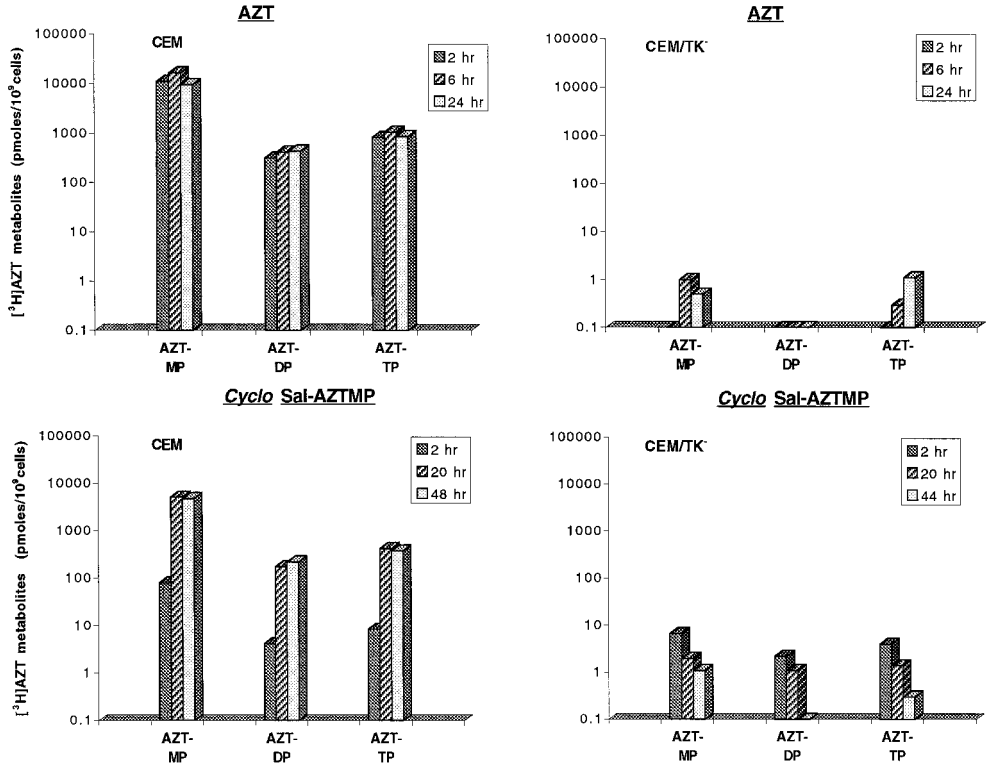


Fig. 3. Metabolism of [^{3}H]AZT (top) and [^{3}H]*Cyclo*Sal-AZTMP (bottom) in wild-type (CEM/0; left) and TK-deficient CEM/TK$^-$ cell cultures (right) on 2, 6, and 24 h (top panels) or 2, 20, and 44 h (bottom panels) incubation of the cells with radiolabeled drugs at a concentration of 0.07 μM (*Cyclo*Sal-AZTMP) or 0.09 μM (AZT) of the radiolabeled drugs.

study was in agreement with our previous investigations (Saboulard et al., 1999). In this study, a variety of AZTMP and d4TMP phosphoramidate prodrugs were exposed to a rat liver enzyme preparation. All of the AZTMP prodrugs tested showed mainly conversion to free AZT, whereas for the d4TMP prodrugs, formation of the free nucleoside was much less pronounced or even undetectable. Moreover, when AZTMP and d4TMP were directly exposed to the rat liver enzyme preparation, the percentage of hydrolysis of d4TMP was 6 and 35% after 1- and 16-h incubations, respectively, whereas for AZTMP, the percentage hydrolysis was 24 and 70% after 1- and 16-h incubations, respectively (Saboulard et al., 1999).

TABLE 2
Metabolism of [^{3}H]AZT and [^{3}H]*Cyclo*Sal-AZTMP on 24-h incubation of CEM cells at different initial drug concentrations for 24 h
Values are the mean of two of four independent experiments.

Initial AZT Concentration	[CH_3–^{3}H]AZT			
	AZT (fr 2-3)[a]	AZTMP (fr 10-11)[a]	AZTDP (fr 15-16-17)[a]	AZTTP (fr 25-26-27)[a]
μM	*pmol / 10^9 cells*			
0.09	83	8,437	261	444
1	640	41,170	450	860
10	2,800	113,600	590	840
100	34,000	414,000	1,900	2,700

Initial *Cyclo*Sal-AZTMP Concentration	[CH_3–^{3}H]*Cyclo*Sal-AZTMP			
	AZT + Prodrug (fr 2-3)[a]	AZTMP (fr 10-11)[a]	AZTDP (fr 15-16-17)[a]	AZTTP (fr 25-26-27)[a]
μM	*pmol / 10^9 cells*			
0.07	41	4,926	148	331
1	620	36,180	370	650
10	2,500	107,400	650	940
100	32,000	401,000	1,800	1,500

[a] The values within parentheses represent the elution times (min) (or fraction numbers) of the metabolites. Each fraction contains 1 ml, collected in 1 min.

TABLE 3
Metabolites of 0.09 μM [^{3}H]*Cyclo*Sal-AZTMP in CEM, MT-4, Molt 4/C8, DAUDI, PBL, and M/M cells after 24-h incubation
Values are the mean of two to four independent experiments.

Cell Line	[^{3}H]*Cyclo*Sal-AZTMP			
	AZT + Prodrug (fr 2-3)[a]	AZTMP (fr 9-10)[a]	AZTDP (fr 14-15)[a]	AZTTP (fr 22-23)[a]
	pmol / 10^9 cells			
CEM	39	5,5475	236	425
MT-4	64	2,002	267	385
MOLT 4/C8	111	7,854	357	755
DAUDI	58	11,572	781	1,920
PBL (resting)	5.8	2.6	0.33	0.5
PBL (activated)	17	843	22	33
M/M	23	1,044	28	29

[a] The values within parentheses represent the elution times (min) (or fraction numbers) of the metabolites. Each fraction contains 1 ml, collected in 1 min.

TABLE 4
Metabolites of [^{3}H]AZT in PBL cells after 24 h of incubation
Values are the mean of two independent experiments.

Cell Line	[^{3}H]AZT			
	AZT (fr 2-3)[a]	AZTMP (fr 9-10-11)[a]	AZTDP (fr 15-16-17)[a]	AZTTP (fr 24-25-26)[a]
	pmol / 10^9 cells			
PBL	3.0	3.6	0.53	0.57
PBL (activated)	30	2,202	38	44

[a] The values within parentheses represent the elution times (min) (or fraction numbers) of the metabolites. Each fraction contains 1 ml, collected in 1 min.

Discussion

The *Cyclo*Sal nucleotide prodrugs were originally designed to be cleaved by a chemical hydrolysis mechanism involving a successive, coupled cleavage of the phenylester and benzylester of the nucleotide prodrug phosphotriester molecule (Meier, 1998). The preferential cleavage of the phenylester bond is most likely to occur first, and its speed can be controlled by the nature of 3- and 5-substituents in the phenyl part of the prodrug molecule. The resulting 2-hydroxybenzyl phosphodiester is then spontaneously cleaved to yield the nucleotide and the remaining diol (salicyl alcohol; Fig. 4). The chemical $T_{1/2}$ of the 3-methyl-substituted *Cyclo*Sal-AZTMP derivative used in this study (Fig. 1) was determined as being ~0 h in phosphate buffer, pH 7.3 (Meier et al., 1997b). It cannot be excluded, however, that inside some cell types of the body (i.e., liver cells), enzymatic degradation of the *Cyclo*Sal-AZTMP prodrug to AZTMP occurs in addition to chemical hydrolysis of the molecule. If this happened, the release of AZTMP from the prodrug molecule and eventual formation of AZTTP should be the result of the complex interplay of both a chemically and an enzymatically driven process.

The intracellular AZTTP (and AZTMP) formation peaked later in CEM cells when *Cyclo*Sal-AZTMP was administered (24 h) than after AZT administration (6 h). Thus, it seems that TK-dependent phosphorylation of AZT to AZTMP in AZT-treated cells proceeds much faster than the release of AZTMP from *Cyclo*Sal-AZTMP by the two-step prodrug hydrolysis. In addition, the *Cyclo*Sal-AZTMP-derived levels of AZTTP, the antivirally active metabolite of AZT, were slightly lower than the AZT-derived AZTTP levels (~50% at 24, 48, and 72 h), an observation that is in agreement with the antiviral activity of AZT and *Cyclo*Sal-AZTMP in CEM cells (EC_{50} = 0.005–0.006 and 0.006–0.013 μM, respectively; Meier et al., 1997b, 1998a). The comparable antiviral potencies of AZT and *Cyclo*Sal-AZTMP also indicate that the antiviral activity of these compounds is mainly determined by the intracellular AZTTP levels obtained at, or after, 24 h rather than the AZTTP levels generated before this time point. In fact, time-of-addition experiments in which nucleoside reverse transcriptase inhibitors are added at different time points after virus infection revealed that there is a marked lag period before the reverse transcriptase process starts during the infection process (Witvrouw et al., 1997). These observations are in agreement with our metabolic findings.

It is interesting to note that an intermediate metabolite (i.e., the benzyl phosphodiester derivative of AZTMP) cannot be detected at measurable levels in the cell extracts. This is in sharp contrast with the marked intracellular accumulation of the intermediate metabolite (i.e., alaninylphosphoramidate d4TMP diester) in the case of the APA prodrug of d4TMP (Balzarini et al., 1996). Thus, the conversion (spontaneous reaction) of the intermediate metabolite to AZTMP (and/or AZT) must occur rapidly and is clearly not rate limiting to obtain the free nucleoside or nucleotide (Fig. 4). Also, the initial intracellular appearance of AZTMP in cells exposed to *Cyclo*Sal-AZTMP is markedly lower than that in AZT-treated cells. This suggests that cellular uptake of the

intact prodrug molecule is slower than that of AZT and/or that the chemical or enzymatic conversion of the prodrug to AZTMP is a relatively slow process (chemical $T_{1/2} \sim 10$ h). Although the first hypothesis cannot be completely excluded, it may be rather unlikely because the prodrug, due to its high lipophilicity, should be taken up by passive diffusion (as is also the case for AZT; Zimmerman et al., 1987), rather than by a carrier-mediated transport. However, given the partition coefficients of *Cyclo*Sal-AZTMP and AZT of 26.9 and 1.09, respectively (Meier et al., 1998a), it may not be unlikely that the *Cyclo*Sal-AZTMP prodrug may be retained longer in the cell membrane than AZT due to its much higher lipophilicity.

The low levels of AZTMP and AZTTP in the TK-deficient CEM/TK$^-$ cells exposed to *Cyclo*Sal-AZTMP correlate with the observed poor antiviral efficacy of this prodrug in CEM/TK$^-$ cells (anti-HIV-2 activity of *Cyclo*Sal-AZTMP in CEM/0 and CEM/TK$^-$ cells: 0.013 and 15 μM, respectively; Meier et al., 1997b, 1998a). This is in sharp contrast with the *Cyclo*Sal prodrug of d4TMP that retained full antiviral activity in CEM/TK$^-$ cells (Meier et al., 1997b) and that was found to generate substantial intracellular amounts of d4TMP, d4TDP, and d4TTP in CEM/TK$^-$ cells (data not shown). Thus, our findings demonstrate that AZTMP either is not efficiently released from the prodrug molecule or is efficiently released but rapidly degraded to AZT as soon as it is formed, rather than being further anabolized to AZTTP. If the first hypothesis is valid, it means that *Cyclo*Sal-AZTMP is processed intracellularly differently than *Cyclo*Sal-d4TMP. Because the chemical $T_{1/2}$ values of both molecules were found to be identical ($T_{1/2} \sim 10$ h), this phenomenon should clearly be ascribed to an enzymatic cleavage process, which discriminates between the nature of the nucleotide in the prodrug molecule. On the other hand, the second hypothesis that AZTMP is released in the CEM/TK$^-$ cells but more extensively catabolized to AZT instead of being further anabolized to AZTDP and AZTTP may be equally valid. Indeed, we found that AZTMP is much less stable than d4TMP in freshly prepared CEM cell extracts and that AZTMP is quickly converted to its free nucleoside AZT in the CEM cell extracts. If this phenomenon also occurs in the intact cell, it may be that AZTMP release from the prodrug occurs to a comparable extent in both wild-type CEM and CEM/TK$^-$ cells and that in both cell lines AZTMP is rapidly converted to AZT, giving it no chance to accumulate for further conversion to the 5′-diphosphate and 5′-triphosphate metabolite. The free nucleoside is then again subject to rapid conversion to AZTMP by TK in wild-type CEM cells but not in CEM/TK$^-$ cells that lack the cytosolic TK.

The fact that AZTMP markedly accumulates on exposure of wild-type CEM cells to AZT or *Cyclo*Sal-AZTMP suggests that the anabolism of AZT to AZTMP by cytosolic TK occurs faster than the catabolism of AZTMP to AZT by phosphatases or 5′-nucleotidases. This is not surprising, because cellular

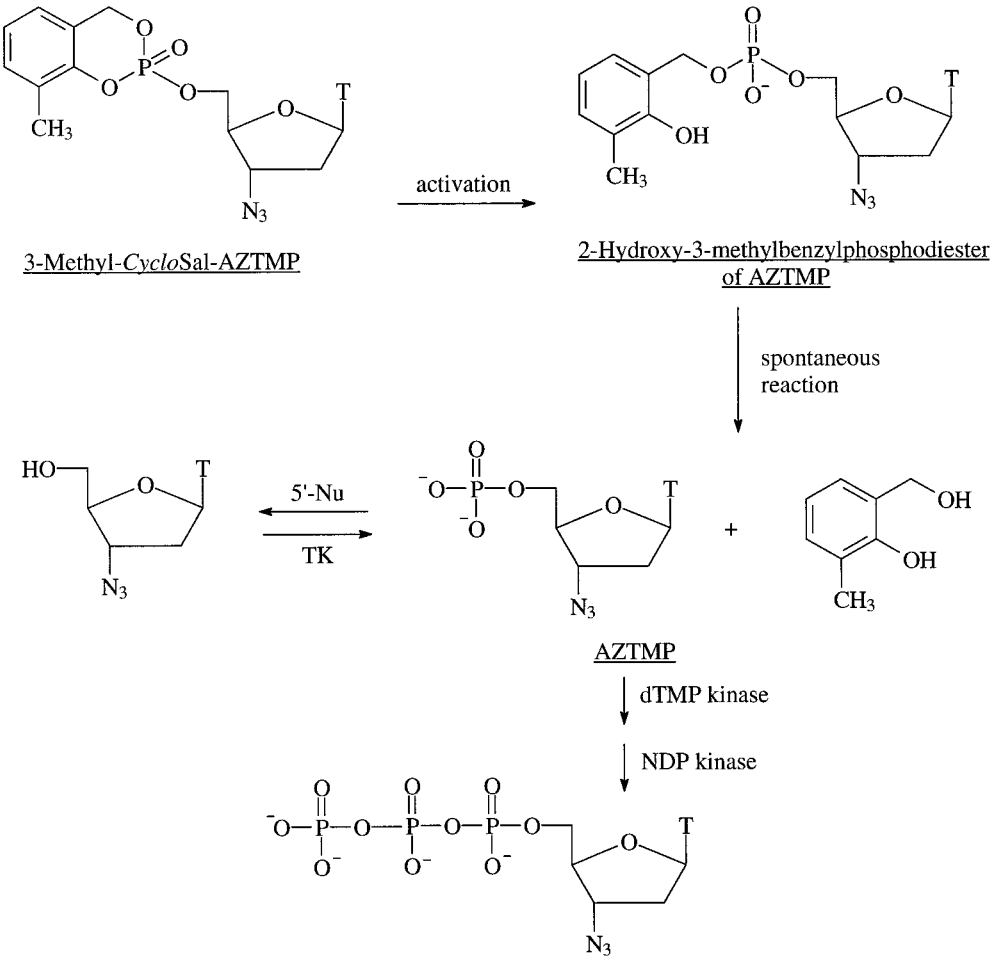


Fig. 4. Proposed activation scheme of *Cyclo*Sal-AZTMP.

TK has a strong affinity and high catalytic conversion rate for AZT (K_m and V_{max} for AZT are comparable to the values for the natural substrate thymidine; Furman et al., 1986; Balzarini et al., 1989). In contrast, thymidylate kinase has a good affinity for AZTMP (K_m = 8 versus 4 μM for dTMP) but a very low V_{max} value (0.3% of that of dTMP; Furman et al., 1986), resulting in a substantial retardation of further anabolism of AZTMP to its diphosphates and triphosphates. It remains to be determined which enzyme is responsible for the rapid conversion of AZTMP to its free nucleoside. It is reasonable to assume that the relatively slow release of AZTMP from the *Cyclo*Sal-AZTMP prodrug molecule contributes to the rapid conversion of the majority of AZTMP molecules to AZT as soon as they are formed in the cells. Therefore, we may hypothesize that an intracellular equilibrium must be rapidly established between dephosphorylation of AZTMP to AZT and the TK-driven phosphorylation of AZT to AZTMP. Both processes must occur at a considerable speed, explaining why very low levels of AZTMP are recorded in *Cyclo*Sal-AZTMP-exposed CEM/TK$^-$ cells, in which the TK-driven phosphorylation is virtually absent due to the TK deficiency of these cells.

It is notable that in M/M, *Cyclo*Sal-AZTMP is eventually converted to a markedly lower extent to AZTTP than in T- and B-lymphocyte cell lines. These data, however, support our view that AZTMP, as soon as it is released from the *Cyclo*Sal prodrug, is quickly converted to AZT. Indeed, macrophages have TK levels that are markedly lower than lymphocytes (i.e., 5.7 pmol formed dTMP/mg protein/min in macrophages compared with 407 pmol formed dTMP/mg protein/min in lymphocyte H9 cells; Perno et al., 1988). Thus, it is conceivable that the eventual formation of AZTTP in prodrug-treated M/M is relatively low compared with lymphocytes when AZTMP released from the prodrug is mainly converted to AZT before back-conversion to AZTMP by cytosolic TK.

In conclusion, we investigated the metabolism of the *Cyclo*Sal-AZTMP phosphotriester prodrug compared with that of the parent AZT in both wild-type CEM and CEM/TK$^-$ cell lines. This revealed the pharmacological basis for the differential anti-HIV activity of this prodrug and its parent nucleoside AZT and for their dramatically reduced antiviral efficacy in TK-deficient CEM/TK$^-$ cells. We also revealed the much higher instability of AZTMP than d4TMP in cell extracts, which may be the pharmacological basis of the failure of *Cyclo*Sal-AZTMP to be active in CEM/TK$^-$ cells. The observed metabolic features are in full agreement with the antiviral data obtained previously (Meier et al., 1997b, 1998a). The study revealed that any pronucleotide approach on AZTMP to efficiently form 5′-diphosphate and 5′-triphosphate metabolites directly derived from the released AZTMP is likely to fail. In this respect, AZT may not be the only nucleoside in this position. Each new nucleoside included in the prodrug approach must be investigated on a case-by-case basis. Resistance of the nucleotide 5′-monophosphates to hydrolytic cleavage in cell extracts may provide good evidence for the eventual successful kinase bypass of their corresponding prodrugs.

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