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THYMIDINE 5′-*O*-PIVALOATE

A PRODRUG DERIVATIVE OF THYMIDINE WITH POTENTIAL APPLICATIONS IN HIGH-DOSE METHOTREXATE THERAPY

WILLIAM D. ENSMINGER and ANDRE ROSOWSKY*

Division of Medicinal Chemistry and Pharmacology, Sidney Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, U.S.A.

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Abstract—Thymidine 5′-*O*-pivaloate was evaluated as a prodrug derivative of thymidine for possible use in the prevention of toxicity from high-dose 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid (methotrexate, MTX) therapy. Plasma levels of free thymidine were higher and remained elevated for a longer period in animals receiving the ester than in animals receiving an equivalent amount of thymidine itself. The higher concentration × time achieved with the ester was reflected in more extensive labeling of bone marrow and gut DNA, and resulted in increased survival of L1210 leukemic mice treated with high-dose MTX. The rate of cleavage of the ester to free thymidine was significantly lower in human serum *in vitro* than in mouse serum.

The use of thymidine as a means of preventing or reversing the toxic effects of methotrexate (MTX)† has been studied in several laboratories *in vitro* [1–5] and *in vivo* [6–12], and the biochemical and pharmacologic rationale of this approach has been reviewed recently [13]. A disadvantageous property of thymidine from a clinical standpoint is its very short plasma half-life (8–10 min), reflecting rapid catabolic breakdown in the liver to thymine and eventually to β-aminoisobutyric acid and CO_2. As a result, in order to maintain an adequate tissue level for a sufficient period of time, it becomes necessary to give the drug in large doses by continuous infusion [11, 12]. One method of circumventing this problem that has been explored in laboratory animals but is cumbersome in human patients is subcutaneous implantation of thymidine-containing cholesterol pellets [6, 7, 14]. In an extension of this approach, we have been interested in the possibility that administration of certain types of prodrug derivatives of thymidine may be superior to high-dose continuous infusion or the use of cholesterol pellets as a form of treatment [15]. In this paper, we report the initial result of a pharmacologic study employing the prototype compound thymidine 5′-*O*-pivaloate (Fig. 1), a sterically hindered ester originally synthesized for an unrelated purpose by Weimann and Khorana [16]. Plasma levels of thymidine were significantly higher, and remained elevated for a longer period of time when thymidine was given to mice as the 5′-*O*-pivaloate than when it was given as the free nucleoside. A higher amount of thymidine was incorporated into the bone marrow and gut DNA of mice when the compound was administered as the prodrug. Finally, L1210 leukemic mice treated with thymidine 5′-*O*-pivaloate were able to withstand a higher dose of MTX than mice treated with an equivalent dose of thymidine, and protection was achieved without loss of antitumour effectiveness.

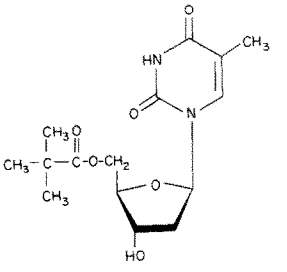

Fig. 1. Structure of thymidine 5′-*O*-pivaloate.

MATERIALS AND METHODS

Unlabeled thymidine and 5-bromo-2′-deoxyuridine were obtained from the Sigma Chemical Corp. (St. Louis, MO), pivaloyl chloride from the Aldrich Chemical Co. (Milwaukee, WI), and [methyl-^{3}H]thymidine (sp. act. 2 Ci/m-mole) from the New England Nuclear Corp. (Boston, MA). Other chemicals and solvents were of analytical grade. Thin-layer chromatography (t.l.c.) analyses were performed on silica gel (Eastman 13181 with fluorescent indicator) or silicic acid impregnated glass microfiber sheets (Gelman Type SAF). Column chromatography was carried out on silica gel (Baker 5-3405, 60–200 mesh). For the high-pressure liquid chromatography (h.p.l.c.) experiments, a Waters model ALC-202 instrument equipped with an M6000 pump, U6K injector, and prepacked μBondapak CN column (0.39 × 30 cm) was used. Water for the preparation of h.p.l.c. buffers was deionized by passage through an ion-exchanger (Filterite Corp., Timonium, MD), degassed by boiling, and filtered through a

* To whom reprint requests should be addressed.

† The following abbreviations are used: MTX = methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid); ILS = increase in lifespan; DMSO = dimethylsulfoxide; PBS = phosphate-buffered saline; BUdR = 5-bromo-2′-deoxyudiridine; and c.p.m. = counts/min.

0.2 μm cellulose membrane (Gelman Type GA-6, 25 mm diameter). Nitrocellulose filters used in the final clarification of deproteinated serum samples were from the Millipore Corp. (Bedford, MA). Mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were fed a standard laboratory diet.

Synthesis of thymidine 5′-O-pivaloate. A solution of thymidine (10 g, 0.041 mole) in dry pyridine (50 ml) was added with stirring to an ice-cold solution of pivaloyl chloride (5.1 g, 0.042 mole) in dry pyridine (25 ml). After 4 hr at room temperature, the reaction mixture was evaporated to dryness under reduced pressure. The residue was taken up in methylene chloride, and the solution was extracted successively with 1% H_2SO_4, 5% $NaHCO_3$, and water. Drying over anhydrous Na_2SO_4 and rotary evaporation yielded a white solid which was purified by column chromatography on silica gel (140 g) using $CHCl_3$, 98:2 $CHCl_3$–EtOH, and 98:5 $CHCl_3$–EtOH as eluents. Individual fractions (8 ml) that were t.l.c.-homogeneous (silicic acid, 9:1 $CHCl_3$–MeOH, R_f 0.71) were pooled and evaporated, and the residue was recrystallized from benzene to obtain colorless crystals (7.9 g, 59 per cent yield); m.p. 97–98°, resolidifying and melting again at 125–127° (lit. [16] m.p. 96°, 140–141°).

Anal. Calc for $C_{15}H_{22}N_2O_6$: C, 55.20; H, 6.80; N, 8.58. Found: C, 55.14; H, 7.04; N, 8.31.

For the preparation of radiolabeled thymidine 5′-*O*-pivaloate, unlabeled thymidine (1.6 g, 0.0066 mole) was added to [methyl-^{3}H]thymidine (1.2 mg, 10 mCi) and the esterification reaction was carried out as described above.

Pharmacokinetics of thymidine and thymidine 5′-O-pivaloate in the mouse. Normal BDF_1 male mice were treated with [^{3}H]thymidine (sp. act. 2 mCi/m-mole, 12 mg/mouse) in water or [^{3}H]thymidine 5′-*O*-pivaloate (sp. act. 2 mCi/m-mole, 16 mg/mouse) in 10% Tween 80. The drugs were administered by subcutaneous injection or orally via a plastic tube. At measured time intervals (1, 4 and 6 hr), blood was drawn by cardiac puncture and 100-μl aliquots of plasma were analyzed by scintillation counting. Two mice were used in each experiment for each time point.

In the case of mice receiving [^{3}H]thymidine 5′-*O*-pivaloate, aliquots of plasma were diluted with 2 vol. of MeOH, the precipitated proteins were spun down at 0° (2000 rev/min, 6 min), and after adding unlabeled thymidine and thymidine 5′-*O*-pivaloate as carriers, the supernatant fractions were spotted on silica gel t.l.c. sheets which were developed with 9:1 $CHCl_3$–MeOH. Spots were visualized under ultraviolet light (254 nm) and cut out with scissors, and the radioactivity was determined. Control experiments using known amounts of labeled compounds indicated that the counting efficiency was 25–30 per cent. The extent of bioconversion of thymidine 5′-*O*-pivaloate to thymidine was estimated by calculating the (nucleoside/nucleoside + nucleoside ester) ratio for at least ten aliquots of plasma at each time point and obtaining an average.

Labeling of DNA by thymidine and thymidine 5′-O-pivaloate. Animals from the preceding experiment were killed by cervical dislocation, their femurs were removed, and the marrow was flushed from the bone with 2 ml PBS. The cells were spun down at 0° (2000 rev/min, 6 min), washed with PBS, and resuspended in PBS (1 ml). The radioactivity in a 50-μl aliquot was determined by scintillation counting. The DNA content in the marrow was obtained by digesting a sample in 0.1 N NaOH overnight at 37° and applying the diphenylamine/acetaldehyde method of Burton [17]. For the analysis of thymidine incorporation into gut DNA, 2 cm of the most proximal section of the small bowel was removed from each animal, the bowel was flushed with PBS (2 ml) and minced, and a 50-μl aliquot of the homogenate was counted. The DNA content was determined as above. The specific activity at each time point was expressed as counts/min/μg of DNA.

Comparison of thymidine and thymidine 5′-O-pivaloate in the MTX treatment of L1210 leukemic mice. Groups of seven BDF_1 mice were implanted intraperitoneally with 10^5 L1210 cells on day 0, and drugs were administered by intraperitoneal injection starting on day 1. MTX (100, 200, 300 mg/kg single dose) and thymidine (500 mg/kg twice daily for 3 days) were given in water, and thymidine 5′-*O*-pivaloate (1000 mg/kg once daily, or 500 mg/kg twice daily, for 3 days) was given in DMSO (0.2 ml). The effects of the two rescue agents were assessed on the basis of the median survival at each dose of MTX.

Cleavage of thymidine 5′-O-pivaloate by mouse serum and human serum. A 30-μl aliquot of 0.1 M thymidine 5′-*O*-pivaloate in DMSO was added to 3 ml of fresh whole serum which was incubated at 37°. At periodic time intervals (0.5, 1, 2, 4 and 24 hr), 0.5 ml serum was removed and 10 μl of 0.01 M BUdR was added as an internal standard for h.p.l.c. Deproteinization was achieved by adding 0.75 ml of cold MeOH, storing overnight at 4°, centrifuging at 0° (2000 rev/min, 6 min), and clarifying the supernatant fraction by passage through a 0.2 μm nitrocellulose filter fitted to the end of a syringe. The amount of thymidine in the sample was determined by h.p.l.c. analysis, as described below.

Standard solutions of thymidine (0.5, 0.8, 1.5 and 2.0×10^{-4} M) and BUdR (0.8×10^{-4} M) were prepared in serum which had been deproteinized previously by precipitation with MeOH. The elution buffer consisted of ammonium phosphate (0.532 g, 0.004 mole) dissolved in a mixture of water (800 ml) and ethanol (80 ml) which was adjusted to pH 8 with ammonia. The flow rate through the column was maintained at 1.0 ml/min. A calibration curve was obtained by making triplicate injections of 3-μl aliquots of the standard solutions and plotting the TdR/BUdR peak height ratio as a function of TdR concentration. The calibration data had a calculated correlation coefficient of 0.999, and the standard deviations ranged from ± 0.001 to ± 0.025. In order to determine how much thymidine was produced on incubation of thymidine 5′-*O*-pivaloate in mouse and human serum, 3-μl aliquots of the MeOH deproteinized test samples (see above) were chromatographed, and the observed TdR/BUdR peak height ratios were converted into thymidine concentrations from the linear calibration curve or by regression analysis using a programmable calculator. Results were expressed as per cent of the theoretical yield based on an initial concentration of 1×10^{-4} M for thymidine 5′-*O*-pivaloate.

RESULTS

Subcutaneous administration of equivalent amounts

Table 1. Pharmacokinetics of [^{3}H]thymidine and [^{3}H]thymidine 5′-*O*-pivaloate in the mouse

Compound	Route*	cpm/100 μl Plasma† 1 hr	4 hr	6 hr
Thymidine	s.c.	22,800	12,100	4,800
Thymidine 5′-*O*-pivaloate	s.c.	54,000 (75)	301,000 (87)	47,000
Thymidine	p.o.	3,400	8,790	
Thymidine 5′-*O*-pivaloate	p.o.	100,000 (80)	118,000 (19)	

* Abbreviations: s.c. = subcutaneous; p.o. = oral.
† Values in parentheses represent % thymidine based on t.l.c. analysis.

Table 2. DNA labeling with [^{3}H]thymidine and [^{3}H]thymidine 5′-*O*-pivaloate in normal mouse

Compound	Route*	Specific activity (cpm/100 μg DNA) Bone marrow 1 hr	4 hr	6 hr	Gut 1 hr	4 hr	6 hr
Thymidine	s.c.	30	44	54	7	10	23
Thymidine 5′-*O*-pivaloate	s.c.	270	1500	1700	60	170	420
Thymidine	p.o.	20	60		9	24	
Thymidine 5′-*O*-pivaloate	p.o.	430	960		70	250	

* Abbreviations: s.c. = subcutaneous; p.o. = oral.

Table 3. Treatment of L1210 mouse leukemia with MTX plus thymidine or thymidine 5′-*O*-pivaloate*

MTX (mg/kg)	Thymidine or ester	Median survival (days)	% ILS	Toxic deaths
None	None	8.5 ± 0.9		
100	None	12.6 ± 2.3	+48	1/7
200	None	10.4 ± 2.7	+22	2/7
300	None	7.0 ± 1.5	−18	6/7
200	Thymidine, b.i.d. × 3	12.6 ± 2.3	+48	1/7
200	Thymidine 5′-*O*-pivaloate, b.i.d. × 3	15.0 ± 1.2	+76	0/7
200	Thymidine 5′-*O*-pivaloate, q.d. × 3	13.4 ± 1.0	+48	0/7
300	Thymidine, b.i.d. × 3	6.1 ± 0.9	−28	6/7
300	Thymidine 5′-*O*-pivaloate, b.i.d. × 3	17.1 ± 1.3	+101	0/7 $P < 0.01$
300	Thymidine 5′-*O*-pivaloate, q.d. × 3	15.7 ± 1.0	+85	0/7 $P < 0.01$

* Groups of seven BDF_1 mice were injected i.p. with 10^5 L1210 cells on day 0, and treatment was started on day 1: MTX, i.p. in water; thymidine, i.p. in water (1000 mg/kg/day) twice daily at 9:00 a.m. and 5:00 p.m. for 3 days; thymidine 5′-*O*-pivaloate, i.p. in DMSO (1000 mg/kg/day) twice daily at 9:00 a.m. and 5:00 p.m. for 3 days or once daily for 3 days. % ILS = (T/C-1 100. Differences in % ILS between the b.i.d. × 3 and q.d. × 3 schedule for thymidine 5′-*O*-pivaloate were not found to be statistically significant at either 200 or 300 mg/kg of MTX.

of labeled thymidine or thymidine 5′-*O*-pivaloate to mice resulted in markedly different pharmacokinetics, as evidenced by the data in Table 1. Total plasma radioactivity (c.p.m./μl) was 2.4-fold higher after 1 hr when thymidine was given as the ester than when it was given as the free nucleoside, and after 4 hr this ratio increased to 25:1. Of the label present in plasma at these time points, 25 and 13%, respectively, consisted of unchanged thymidine 5′-*O*-pivaloate, according to t.l.c. analysis. Somewhat similar results were obtained when the two drugs were administered via the oral route. After 1 hr there was 30 times more plasma radioactivity with the ester than with the parent nucleoside, and after 4 hr this difference was still 13-fold. On the other hand, there were significant variations with respect to plasma thymidine as a function of time following subcutaneous vs oral administration. Whereas 87% of the radioactivity at 4 hr consisted of free thymidine when the ester was given subcutaneously (t.l.c. analysis), only 19% represented free thymidine when the ester was given orally. After 1 hr, however, free thymidine accounted for about the same fraction (75–80%), irrespective of the route of administration.

Table 2 shows the incorporation of labeled thymidine into bone marrow and gut of mice treated with thymidine vs thymidine 5′-*O*-pivaloate. Following subcutaneous administration, there was a 9-fold higher uptake of radioactivity into bone marrow after 1 hr and a 34-fold higher uptake after 4 hr with thymidine 5′-*O*-pivaloate than with thymidine. After oral administration, labeling of bone marrow DNA was 21-fold higher at 1 hr and 16-fold higher at 4 hr, and the same qualitative pattern was observed in the labeling of gut DNA except that the amount of radioactivity taken up

by the latter tissue was lower at the two time points selected for comparison.

The results of an experiment comparing protection from MTX toxicity with thymidine vs thymidine 5′-*O*-pivaloate are presented in Table 3. A single 300 mg/kg dose of MTX was toxic to the entire group of seven animals (toxicity being defined as death occurring sooner than in untreated controls, in this instance prior to day 8). Some toxicity was likewise seen at 200 mg/kg. Injection of thymidine (1000 mg/kg/day) twice daily for 3 days starting on the same day as MTX treatment brought about protection at the lower but not the higher MTX dose, whereas injection of thymidine 5′-*O*-pivaloate (1000 mg/kg/day) on the same schedule achieved protection at both MTX doses. A twice-daily injection did not bring about a markedly higher ILS than injection of a single daily dose. A single MTX dose of 100 mg/kg produced an increase in mean lifespan of 48% without protection. When the MTX dose was 200 mg/kg, thymidine and thymidine 5′-*O*-pivaloate were approximately equal in their ability to protect the animals. No toxicity was observed on treatment with either an equivalent dose of pivalic acid (the expected cleavage product from thymidine 5′-*O*-pivaloate) or with DMSO, and no increase in survival was achieved by giving nucleoside ester without MTX (data not shown).

A comparison of the rate of cleavage of thymidine 5′-*O*-pivaloate to free thymidine in whole mouse vs human serum *in vitro* is given in Fig. 2. After 1 hr of incubation at 37° in mouse serum, 65% of the theoretical yield of thymidine was already present. This increased to 75–80% at 2 hr, and remained approximately constant for 24 hr. In whole human serum on the other hand, less than 10% of the theoretical yield of thymidine was present after 1 hr, and even after 4 hr this only increased to 30%. After 24 hr the amount of thymidine in mouse and human serum became comparable. In a control experiment performed at 37° in phosphate-buffered saline (pH 7.4), the amount of thymidine released from the ester was < 5% after 24 hr. In every instance, t.l.c. analysis confirmed that drug not converted into thymidine was still present as the 5′-*O*-pivaloate.

DISCUSSION

Several noteworthy differences between thymidine and thymidine 5′-*O*-pivaloate were observed in this study. It is evident from the data in Table 1 that the use of a prodrug derivative such as the 5′-*O*-pivaloate ester permits a higher C × T (concentration × time) to be achieved for thymidine than is possible by giving an equivalent of thymidine itself, either orally or subcutaneously. The route of administration of thymidine 5′-*O*-pivaloate also appeared to have a marked effect on the pharmacokinetics. When labeled ester was given subcutaneously, the radioactivity in the plasma was lower at 1 hr than when the ester was given orally, whereas at 4 hr the opposite was observed. The data support the view that oral absorption of the ester is efficient and leads to high initial plasma levels with rapid clearance, while distribution of the drug from a subcutaneous depot is a more gradual process giving rise to lower initial plasma levels and relatively slow clearance. It should be noted, however, that the analysis of the pharmacokinetic data is complicated since the measured radioactivity in the plasma always represents a mixture of ester and parent nucleoside.

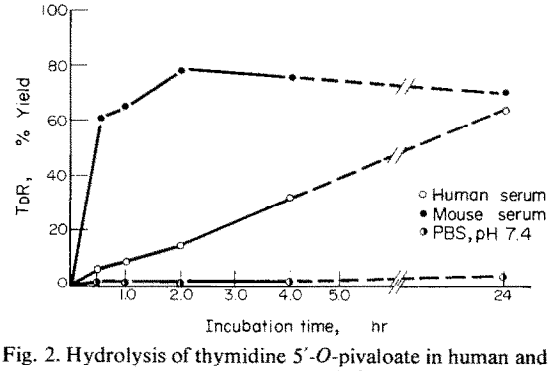

Fig. 2. Hydrolysis of thymidine 5′-*O*-pivaloate in human and mouse serum at 37°.

One of the anticipated outcomes of using a prodrug derivative to increase the C × T of thymidine was an increase in labeling of the DNA in proliferative tissues, such as bone marrow and gut. The results in Table 2 demonstrate that the 5′-*O*-pivaloate ester was in fact 10–30 times more effective than the parent nucleoside in labeling bone marrow DNA in normal mice over a 6-hr period, and 10–20 times more effective in labeling gut DNA. Thymidine was taken up more extensively into bone marrow DNA than gut DNA, and the same order was observed when the ester was given in place of the parent nucleoside. At 1 hr, incorporation into bone marrow DNA appeared to be somewhat favored when the ester was given orally, but after 4 hr there was greater labeling following subcutaneous administration. These results were consistent with the pharmacokinetic findings (Table 1) that the oral route produces higher plasma levels of drug than the subcutaneous route only at the 1 hr time point. Our observation that labeling of gut DNA was less extensive than labeling of marrow DNA can be explained on the basis that mouse intestinal cells are less efficient than mouse marrow in utilizing the thymidine salvage pathway, and is consistent with the well-known fact that gut rather than marrow toxicity is MTX dose-limiting in the mouse [18].

Since we had established that the use of thymidine 5′-*O*-pivaloate in place of thymidine leads to significantly increased DNA labeling of normal mouse tissues, it was not surprising to find the ester to be superior to the parent nucleoside in protecting L1210 leukemic mice from high-dose MTX toxicity (Table 3). It has been reported previously that the toxic effects of a single 300 mg/kg dose of MTX can be prevented by giving 500 mg/kg of thymidine three times a day for 3 days, i.e. a total dose of 4500 mg/kg [6, 7]. In the present study, we found that reducing the amount of thymidine to two 500 mg/kg doses/day for 3 days (3000 mg/kg total) was ineffective. On the other hand, the animals could be protected, with no loss of antitumor effect, with a single 1000 mg/kg dose of thymidine 5′-*O*-pivaloate daily for 3 days (equivalent to a total dose of free thymidine of 2250 mg/kg). Thus, by using the prodrug form of thymidine we were able to reduce the total amount of protecting agent by 50 per cent, with the added practical convenience of a single daily injection.

The high thymidine levels that were achieved in the plasma by giving thymidine 5′-*O*-pivaloate are presum-

ably due to the fact that the ester is less susceptible to the cleaving action of thymidine phosphorylase. Decreased cleavage could be due either to an inherent lack of affinity for the phosphorylase enzyme, as has been noted for some 5′-O-substituted derivatives of thymidine [19], or to the ability of the drug to interact with plasma proteins. A similar interpretation has been given by other workers to account for the greater stability and improved therapeutic effect of esters of 5-fluoro-2′-deoxyuridine (FUdR) [20] and 6-azauridine [21].

An inherent limitation to the use of ester derivatives as prodrugs in rodent systems is that these animals possess high levels of serum esterase [22]. Because of this, it is difficult if not impossible for the therapeutic benefits of a prodrug such as thymidine 5′-O-pivaloate to be tested adequately in the mouse. It is known, on the other hand, that esterase activity is relatively deficient in man [23, 24], and our finding that thymidine 5′-O-pivaloate was hydrolysed only 30 per cent after 4 hr in human serum as compared to 75 per cent in mouse serum is consistent with this fact. One would predict on this basis that, if the 5′-O-pivaloate or some other ester of thymidine were tested in man, its half-life as a prodrug ought to be more prolonged than in mice and rats.

Preliminary studies with other esters of thymidine have been carried out in our laboratory. An example is the 5′-O-adamantoate derivative [25], which behaved comparably to the 5′-O-pivaloate in protecting L1210 leukemic mice from MTX toxicity and being cleaved to thymidine by serum esterases (A. Rosowsky and M. H. N. Tattersall, unpublished results). Other esters of thymidine, as well as other types of functional derivatives, are under investigation with a view to the development of orally acceptable prodrugs than can replace continuous infusion of large amounts of free nucleoside as a means of counteracting the toxic effects of high-dose MTX therapy in man.

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