Disposition and Oral Bioavailability in Rats of an Antiviral and Antitumor Amino Acid Phosphoramidate Prodrug of AZT-Monophosphate

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Purpose. The purpose of this study was to characterize the *in vivo* disposition of 3′-azido-2′-deoxythymidine-5′-methylamino-L-tryptophanylphosphoramidate (NMe-Trp-AZT), a potential pronucleotide of 3′-azido-2′-deoxythymidine monophosphate (AZT-MP).

Methods. The *in vitro* metabolic stability of NMe-Trp-AZT was evaluated in a wide variety of tissue homogenates. NMe-Trp-AZT was administered orally ($n = 3$) to female Sprague–Dawley rats. Its biliary excretion and intestinal permeability were also studied.

Results. Renal excretion of unchanged prodrug (16.4 ± 5.6% of the total dose administered intravenously), its conversion to AZT (12.1 ± 5.4% of total dose administered intravenously), and its biliary excretion (54.3 ± 4.9% of the total dose up to 4 h after intravenous administration) accounted for most of the elimination of NMe-Trp-AZT. Significant amounts of AZT were found in both plasma and urine after oral administration of the prodrug. The prodrug itself was not permeable through the small intestinal wall but was slowly converted to AZT-MP in gastric fluids at low pH.

Conclusions. The NMe-Trp-AZT prodrug itself was not orally bioavailable because of poor intestinal permeability; however, AZT was readily available in the systemic circulation after the oral administration of the prodrug. Modification of the phosphoramidate to promote intestinal uptake should lead to enhanced oral bioavailability of this and other nucleoside phosphoramidate monoesters.

KEY WORDS: AZT; prodrugs; pronucleotide; pharmacokinetics; biliary excretion; oral bioavailability; intestinal permeability.

INTRODUCTION

Nucleoside analogues have shown great success in the treatment of cancer and viral infection (1,2). After activation through intracellular phosphorylation, nucleoside analogues mimic their naturally existing nucleoside counterparts and are incorporated into the DNA strand in virus-infected or cancer cells. Subsequently, they effectively interrupt DNA synthesis in the virus and target cells (1). Amino acid phosphoramidates have been shown to be promising prodrugs for nucleoside monophosphates (3–5). Delivering the monophosphate directly to the cells bypasses the initial rate-limiting phosphorylation step of the nucleoside. The down-regulation of this phosphorylation step has been shown to be one mechanism for resistance (6). Recently, it has been shown that amino acid phosphoramidate monoesters of AZT have exhibited both potent *in vitro* anti-HIV-1 activity and selective anti–breast cancer activity (7–10). Studies have been conducted to evaluate the *in vitro* plasma stability and protein binding as well as to characterize the *in vivo* pharmacokinetic behavior of a series of AZT-monophosphate (AZT-MP) prodrugs in rats (11). In general, the phosphoramidates exhibited significantly longer plasma half-lives and greater tissue distribution than AZT (11) .

Based on the results obtained in those studies, 3′-azido-2′-deoxythymidine-5′-methylamino-L-tryptophanylphosphoramidate (NMe-Trp-AZT, Fig. 1) was identified for further assessment and development. The work presented in this report demonstrates our effort to fully understand the disposition of NMe-Trp-AZT. The results from this research serve as a guide for further optimization of this lead compound by improving its pharmacokinetic characteristics through chemical modification.

MATERIALS AND METHODS

Materials

Female Sprague–Dawley rats (weighing 250–280 g) were purchased from Harlan (Indianapolis, IN). Frozen rat tissues were purchased from Harlan Bioproducts for Science (Indianapolis, Indiana). NMe-Trp-AZT and 3′-azido-2′-deoxythymidine-5′-ethylamino-L-tryptophanylphosphoramidate (NEt-Trp-AZT, the internal standard for HPLC) were prepared as previously described (9). AZT, pepsin, and HEPES were purchased from Sigma (St. Louis, Missouri). Other reagents were of highest grade available.

In Vitro Metabolic Stability Studies

Tissue Homogenate Incubation Studies

Frozen tissues including liver, small intestine, kidney, lung, spleen, and lymph nodes were thawed in the cold room at 4°C, weighed, and washed with ice-cold phosphate-buffered saline (PBS, pH 7.4) to remove residual blood. Tissue homogenates in PBS were prepared in dilutions ranging from 1:2 to 1:10 (w/v) depending on the tissue. The homogenate was centrifuged at 10,000 × *g* for 1 h at 4°C, and the supernatant was used for the incubations.

Three incubations were carried out at 37°C with each tissue homogenate. The final concentration of NMe-Trp-AZT in homogenates ranged from 5 to 50 μM. Serial samples of 100 μl were withdrawn over 90 min after the start of the incubation. Similar studies with freshly prepared liver and small intestine homogenates were conducted with prodrug concentrations of 500–3000 μM and serial sampling up to 8 h.

Stability Studies in Gastric Fluids and Intestinal Contents

The stability of NMe-Trp-AZT was evaluated in artificial gastric fluid at various acidic pH values as well as in intestinal contents. The intestinal contents from three female Sprague–Dawley rats were collected (approximately 10 ml PBS wash

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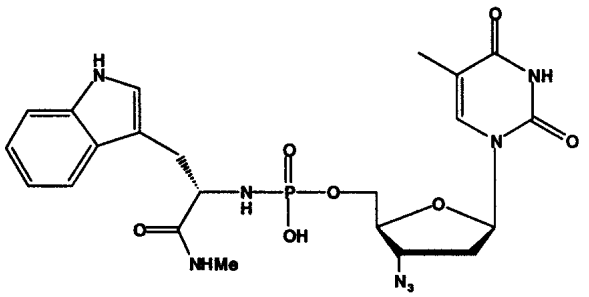

Fig. 1. Chemical structure of 3′-azido-2′-deoxythymidine-5′-methylamino-L-tryptophanylphosphoramidate (NMe-Trp-AZT).

each), combined, and centrifuged at 10,000 × *g* for 1 h at 4°C. The supernatant was used for the incubations.

Artificial human gastric fluid was prepared (12). The pH was adjusted to 2.0, 3.0, or 4.0 with 1 N NaOH solution. Three incubations were prepared at each pH level with a final concentration of NMe-Trp-AZT of 500 to 3000 μM. Samples of 100 μl were withdrawn for up to 8 h.

Animal Studies

Animal studies were conducted in accordance with guidelines set forth in the University of Minnesota Animal Care and Use Manual.

Pharmacokinetics of NMe-Trp-AZT after Oral Dosing

Female Sprague–Dawley rats (n = 3, weighing 250–280 g) received femoral vein and artery cannulas 24–48 h before the administration of the prodrug. A target dose of 380 μmole/kg NMe-Trp-AZT (equivalent to 100 mg/kg of AZT) was administered by gavage. Serial blood samples of 200 μl were drawn from the femoral arterial cannula before dosing and over a 24-h period postdosing. Urine samples were also collected from the metabolic cage before the study and over 24 h postdose.

Biliary Excretion Study

Female Sprague–Dawley rats (n = 3, weighing 250–280 g) were anesthetized with pentobarbital (50 mg/kg i.p.), and the femoral vein and bile duct were cannulated. Blank bile was collected for 1 h. NMe-Trp-AZT dosing solution prepared in saline was administered into the femoral vein at a bolus dose of 190 μmole/kg. Bile was collected into preweighed vials at 1-h intervals up to 4 h postdose.

In Situ Single-Pass Intestinal Perfusion Study

The single-pass *in situ* gut perfusion model was used to determine the intestinal permeability of NMe-Trp-AZT. Female Sprague–Dawley rats (n = 3, weighing 250–280 g) were fasted for 16 h before the study was conducted, with free access to water. The surgery and perfusion study were conducted as previously reported (13).

Analytic Methods

Plasma and urine samples were analyzed using validated HPLC methods (14). The tissue homogenates and intestinal content incubations were thawed at 4°C and extracted with acetonitrile. NEt-Trp-AZT was added as an internal standard. After vigorous vortex mixing for 20 s, the mixture was centrifuged at 13,000 × *g* for 10 min, and the supernatant was removed and evaporated to dryness under a nitrogen stream at 43°C. The residue was then reconstituted in distilled water with 10% acetonitrile and injected onto the HPLC for analysis. Gastric fluid samples were diluted with 10 mM phosphate buffer (pH 7.4) and injected onto the HPLC column directly.

Bile samples were directly injected onto the HPLC after dilution with distilled water. The standard curve was obtained by spiking blank bile with known concentrations of standards.

All intestinal perfusate samples were injected directly onto the HPLC system after being centrifuged at 13,000 × *g* for 5 min. For the tissue homogenates, intestinal contents, bile, and intestinal perfusate, the same mobile phase conditions were used as for the plasma samples analyzed by HPLC with UV detection at 270 nm (14).

Pharmacokinetic Calculations

The oral bioavailability (F) of NMe-Trp-AZT was not determined because of the absence of the prodrug in plasma or urine samples. Because AZT was found in both plasma and urine samples after oral dosing, F_{AZT} (the fraction of the oral dose of the prodrug converted to AZT) could be calculated with the use of the total amount of AZT collected in the urine after oral administration of the prodrug ($Au_{0\rightarrow\infty\,(AZT)}$) (14):

$$F_{AZT} = Au_{0\rightarrow\infty\,(AZT)}/(fe_{(AZT)} \times D) \quad (1)$$

where $fe_{(AZT)}$ is the fraction of AZT eliminated through renal clearance. This value of $fe_{(AZT)}$ was obtained from a separate study where AZT was administered as an IV bolus to female Sprague–Dawley rats (11). $Au_{0\rightarrow\infty\,(AZT)}$ was estimated from urine samples collected up to 24 h postdose under the assumption that the excretion of AZT formed from the prodrug was essentially complete within 24 h. The underestimation of F_{AZT} by this approximation was negligible.

RESULTS

In Vitro Metabolic Stability Studies

NMe-Trp-AZT did not show any appreciable degradation at 37°C over the 90-min incubation period in any tissue homogenate or in the intestinal contents. The stability results were similar for the preparations from freshly harvested or from frozen organs (data not shown).

In the gastric fluids, pH-dependent degradation was observed. The estimates of the first-order degradation rate constant k and its associated t½ at different pH values are summarized in Table I. The rate of degradation of the prodrug in the pH 2.0 gastric fluid was considerably faster than the rates in the pH 3.0 and 4.0 gastric fluids, suggesting an acid-catalyzed chemical hydrolysis. The product of the hydrolysis of NMe-Trp-AZT in gastric fluids was AZT-MP. AZT was not detected.

Oral Bioavailability Study

The plasma concentration–time profile for one of the rats is shown in Fig. 2. Surprisingly, NMe-Trp-AZT could not be detected in any plasma or urine sample, indicating an apparent oral bioavailability of zero for the prodrug itself. How-

Table I. Degradation Rate Constant (k) and Half-Life ($t_{1/2}$) of 1000 μM NMe-Trp-AZT in Gastric Fluids

pH	k (h^{-1})	$t_{1/2}$ (h)
2.0	0.138 (0.130)[a]	5.02
3.0	0.0053 (0.0044)	131
4.0	No degradation	—

[a] Estimate (standard error of estimate). NMe-Trp-AZT was incubated at 37°C in gastric fluids with pH values of 2.0, 3.0, and 4.0. Triplicate samples were withdrawn of various times, and k estimated from the pooled data.

ever, AZT was present in both plasma and urine samples. The plasma levels of AZT declined with a terminal half-life of 2.97 ± 0.43 h, which was much longer than that of AZT after IV dosing (14,15), indicating that AZT's elimination was prolonged by its delayed formation from NMe-Trp-AZT. The fraction of the oral dose of the prodrug converted to AZT (F_{AZT}) was 29.5 ± 24.3%, which was significantly higher than after an IV dose (12.1 ± 5.4%) (11). Despite the low oral bioavailability of the prodrug, it was able to generate a significant amount of AZT in the systemic circulation as well as prolong the biologic half-life of AZT.

Biliary Excretion Study

The prodrug NMe-Trp-AZT was found in the bile samples at extremely high concentrations. AZT was not detected by the analytic methods used because it is excreted into bile as a glucuronide conjugate in rats (15), and the analytic methods used did not quantify the glucuronide. The total dose recovered in the bile as the prodrug up to 4 h postdosing was 54.3 ± 4.9%.

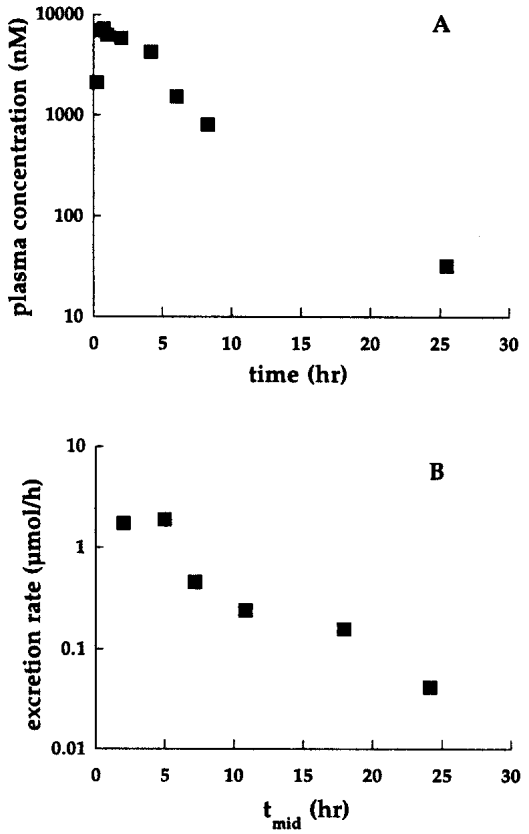

Fig. 2. Pharmacokinetics profiles of AZT in plasma (A) and urine (B) after a single oral dose of 380 μmole/kg of NMe-Trp-AZT in an individual rat. NMe-Trp-AZT was not found in plasma or urine samples.

In Situ Single-Pass Intestinal Perfusion Study

To investigate the role of intestinal permeability on the lack of oral bioavailability of the prodrug, intestinal perfusion studies were carried out. There was little or no absorption of the prodrug within the 120-min perfusion period.

DISCUSSION

The intravenous pharmacokinetics of six phosphoramidate monoester prodrugs of AZT-MP had previously been evaluated, and NMe-Trp-AZT emerged as having the most satisfactory pharmacokinetic characteristics (11) . Because an orally bioavailable prodrug for AZT-MP was desirable, a comprehensive evaluation of the pharmacokinetic properties of NMe-Trp-AZT was carried out. One difficulty that this presented was that the ultimate compound of interest, AZT-MP, is present only intracellularly. If NMe-Trp-AZT had been bioavailable, the next step would have been to evaluate its intracellular delivery and presumed conversion to its active form, AZT-MP.

The plasma concentration–time profile of NMe-Trp-AZT declined in a markedly biphasic manner after a single IV dose in rats, with a long terminal half-life (11.7 ± 0.82 h). The large volume of distribution ($V_{d,ss}$ 15.6 ± 6.0 L/kg) was responsible for the prolonged half-life of the prodrug and indicated that the prodrug would have a significantly larger tissue distribution than AZT. Out of the total dose of the prodrug administered intravenously, 16.4 ± 5.6% was recovered in the urine unchanged, and 12.1 ± 5.4% was converted to AZT (11). This left a significant portion of the dose unaccounted for, and thus, further studies were designed to determine the fate of the remaining portion of the prodrug dose.

Biliary excretion was found to be the major elimination route for NMe-Trp-AZT. More than 50% of the total dose was excreted into the bile unchanged within 4 h after intravenous administration of the prodrug. The pK_a of NMe-Trp-AZT is 0.87 (unpublished data), so the prodrug exists as an anion at physiologic pH. From the polarity and molecular weight of the amino acid phosphoramidate prodrugs of AZT, as well as their ionization under normal physiologic conditions, it is clear that they are excellent candidates for biliary excretion in rats. Overall, the urinary excretion, biliary excretion, and conversion to AZT account for at least 80% of the total intravenous prodrug dose. Because bile samples were collected only up to 4 h after IV dosing, and bile flow decreases with rats under anesthesia, the fraction of the total dose recovered in the bile as the prodrug itself may be underestimated.

After oral dosing, NMe-Trp-AZT itself was not orally bioavailable but was able to generate significant levels of AZT in the systemic circulation. *In vitro* studies were carried out in homogenates of the ′first-pass′ organs—the liver, in-

testine, and intestinal contents—as well as the kidney, spleen, lymph nodes, and lung. NMe-Trp-AZT was found to be relatively stable in all the tissue homogenates tested.

However, the phosphoramidate bond (P–N bond) undergoes hydrolysis in acid. The half-life of NMe-Trp-AZT was 5 h in simulated gastric fluid at a pH of 2.0. AZT-MP (not AZT) was the hydrolysis product. It appears that after oral dosing, the prodrug enters the low-pH environment of the stomach first, where it is partially hydrolyzed to AZT-MP. Both the prodrug and AZT-MP enter the intestine, where AZT-MP may be converted to AZT by phosphatases present in the gut lumen. AZT is known to be rapidly absorbed from the rat intestine and enters the systemic circulation with only minor first-pass hepatic extraction (15). In fact, after IV administration of NMe-Trp-AZT and the other phosphoramidate prodrugs tested, there was always a second peak in the AZT concentration–time profile (11), suggesting substantial enterohepatic recycling. Because of the significant difference in the rate of hydrolysis between pH 2 and 3, the pH of the stomach will have a significant effect on the amount of AZT-MP formed from the prodrug. This could be a source of intersubject variability in AZT absorption.

In the design of the amino acid phosphoramidate prodrugs, it was hoped that this type of compound could take advantage of amino acid or nucleotide transporters in the gastrointestinal tract (16). The low intestinal permeability observed indicates that this is probably not the case. It can also be concluded that because of the low intestinal permeability, enterohepatic recycling will not be important for NMe-Trp-AZT itself even though it is excreted efficiently into the bile.

The low intestinal permeability of the amino acid phosphoramidate monoester prodrugs is an obstacle for the development of orally bioavailable pronucleotides. If these compounds are converted to phosphoramidate diesters, the intestinal permeability could be improved because the increased lipophilicity should allow greater passive diffusion. For this strategy to be successful, however, the compounds would have to be stable on oral dosing but convertible to the corresponding monoester during first-pass metabolism. Further efforts in structural modification are under way to develop pronucleotides with improved oral absorption.

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