Single-Dose Pharmacokinetics and Metabolism of \([^{14}C]\)Remofovir in Rats and Cynomolgus Monkeys

Chin-Chung Lin,* Christine Xu, Nanqun Zhu, David Lourenco, and Li-Tain Yeh

Research and Development, Valeant Pharmaceuticals International, Costa Mesa, California

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Adefovir, 9-(2-phosphonomethoxymethyl)adenine (or PMEA), is an acyclic phosphate analogue of adenine which has been shown to be effective against hepatitis B virus (HBV) in stably transfected human hepatocellular carcinoma cell lines and primary duck hepatocytes infected with duck HBV and in the duck model of hepatitis B (5, 6). PMEA is phosphorylated to PMEA diphosphate by cellular kinases which inhibit HBV DNA polymerase (reverse transcriptase) by competing with the natural substrate dATP and by causing DNA chain termination after its incorporation into viral DNA (9). Cerny et al. (1) reported that PMEA was converted to four metabolites, including PMEA monophosphate and PMEA diphosphate. However, PMEA is poorly absorbed in a number of species, including rats, monkeys, and humans (2, 3, 11). The low oral bioavailability of PMEA appears to be in part a consequence of the limited intestinal permeability of phosphonate, which is ionized at a physiological pH (10).

Adefovir dipivoxil is an oral prodrug of PMEA. In clinical trials, at week 48, serum HBV DNA levels had decreased by a median of 3.52 log copies per ml in the group given 10 mg of adefovir dipivoxil and 2.60 liters/h/kg in monkeys; apparent volume of distribution was 5.99 liters/kg in rats and 2.70 liters/kg in monkeys. Following oral administration, remofovir was extensively converted to 9-(2-phosphonomethoxymethyl)adenine (PMEA) and other metabolites in both species. In rats, excretion of total radioactivity in urine accounted for 61.8% of the i.v. dose and 12.9% of the oral dose, while in monkeys it accounted for 43.5% of the i.v. dose and 34.9% of the oral dose. Following i.v. dosing of \([^{14}C]\)remofovir, fecal excretion of radioactivity accounted for 37.5% of the dose in rats and 17.4% of the dose in monkeys, indicating significant biliary excretion of the drug in animals. PMEA and metabolite A were the major urinary metabolites in both species after i.v. and oral administration of remofovir.

Remofovir, a cyclodiesther prodrug of PMEA, was designed to be efficiently and specifically activated through an oxidative reaction catalyzed by the cytochrome P450 isoenzyme CYP3A4. This activation would result in the generation of a highly charged nucleotide intermediate, which is trapped inside the cells and further converted to the nucleoside triphosphate (M. D. Erion, T. J. Colby, K. R. Reddy, D. A. Mackenna, S. H. Boyer, J. M. Fujitaki, D. L. Linemeyer, D. A. Bullough, and P. D. van Poelje, Abstr. 53rd Ann. Meet. Am. Assoc. Study Liver Dis., abstr. 551, 2002). We recently demonstrated that remofovir was retained and converted to PMEA in the liver (7), following an oral dosing (30 mg/kg of body weight) of \([^{14}C]\)remofovir or \([^{14}C]\)adefovir dipivoxil in rats. Remofovir resulted in 15×-higher liver radioactivity levels than adefovir dipivoxil but only one-third of kidney radioactivity levels (7). Since the liver is the target organ for HBV infection and the kidney is the target for nephrotoxicity, remofovir may provide an opportunity to improve the efficacy and reduce the toxicity that is associated with adefovir dipivoxil.

The aim of this study was to determine the absorption, pharmacokinetics, metabolism, and excretion of remofovir in rats and monkeys.

MATERIALS AND METHODS

Compound. \([5-^{14}C]\)Remofovir (Fig. 1) was synthesized by Moravek Biochemicals, Inc. (Brea, Calif.), by using 8-[\(^{14}C\)]adenine as a precursor. The labeled nucleoside was extensively purified by column chromatography and repetitive recrystallization. The chemical identity and purity were verified by mass spectrometry and proton magnetic resonance spectrometry. The radiopurity (>98%) of the preparation was confirmed by using a high-performance liquid chromatography (HPLC) device coupled with a radioactive detector. Remofovir and PMEA were obtained from Valeant Pharmaceuticals International.

Drug administration and sample collection in rats. Following an overnight fast, male Sprague-Dawley rats received 30 mg/kg of \([^{14}C]\)Remofovir (150 μCi in sterile water for injection, USP, 6 ml/kg) as an intravenous (i.v.) bolus dose via a tail vein (n = 3) or as an oral dose via oral gavage (n = 3). Serial blood samples were collected from three rats for each dose route directly into heparinized Vacutainer tubes and placed in an ice bath. Samples were then immediately centrifuged to harvest plasma. In a separate study, urine samples were collected...
and surrounded by dry ice. Fecal samples were collected at room temperature. All plasma, urine, and fecal samples were stored at −70°C until analyzed. All in-life procedures were in compliance with the Guide for the Care and Use of Laboratory Animals, National Research Council, Institute of Laboratory Animal Resources, 1996.

**Drug administration and sample collection in cynomolgus monkeys.** Following an overnight fast, four male cynomolgus monkey received 30 mg/kg of [14C]remofovir (0.5 mCi in sterile water injection, USP, 2 ml/kg) as an i.v. bolus dose via a saphenous vein or as an oral dose via oral gavage in a crossover design. Serial blood samples from each monkey were collected directly in heparinized Vacutainer tubes and placed in an ice bath. Samples were then immediately centrifuged to harvest plasma. Urine samples were collected from each monkey and surrounded by dry ice. Fecal samples were collected at room temperature. All plasma, urine, and fecal samples were stored at −70°C until analyzed. All in-life procedures were in compliance with the Guide for the Care and Use of Laboratory Animals, National Research Council, Institute of Laboratory Animal Resources, 1996.

**Measurement of radioactivity.** The radioactivity in plasma (0.5 ml) and urine (0.2 ml) was measured using Ultima Gold XR scintillation cocktail and a liquid scintillation counter (model 1900TR; Packard Instrument Company, Meriden, Conn.). Fecal samples were combusted in a sample oxidizer (model 306; Packard Instrument Company) and the resulting 14CO2 was trapped in a mixture of Perma Fluor E and Carbo-Sorb E, followed by liquid scintillation counting. Scintillation counting data were automatically corrected for counting efficiency based on an external standard and an instrument-stored quench curve generated from a series of sealed quenched standards.

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the determination of remofovir and PMEA in plasma.** The analytical method involved the addition of 30 µl of internal standards (250 ng of [13C]remofovir/ml and 1.0 µg of [13C]PMEA/ml), protein precipitation with acetonitrile, solvent evaporation, and reconstitution of residue with a solution (200 µl) of 3% dimethylhexylamine–1.5% acetic acid in 10 mM ammonium acetate. A Discovery C18 column (150 mm by 4.6 mm, 5 µm) was used for remofovir analysis and a YMC PBMN column (100 mm by 3.0 mm, 5 µm) was used for PMEA analysis. For remofovir analysis, the mobile phase contained 55% methanol and 45% 10 mM ammonium acetate (pH 4). For PMEA analysis, the mobile phase contained 20% methanol and 80% 20 mM ammonium acetate (pH 3). The flow rate for both analyses was set at 1.0 ml/min.

A MDS Sciex API 4000 system was used for the analysis. The precursor-to-product ion transitions were monitored at 424 to 151 and 430 to 157 for remofovir and [13C]remofovir, respectively, by using positive electrospray ionization. Each transition was alternately monitored with a dwell time of 250 ms. Collision-activated dissociation gas was set at 10, curtain gas was set at 35 (arbitrary units). The TurboIon Spray (TIS) source temperature was maintained at 650°C and the IonSpray voltage was set at 5,000 V. Both the nebulizer (GS1) and TIS (GS2) were set at 50. Unit resolution was applied for both Q1 and Q3.

The precursor-to-product ion transitions were monitored at 272 to 134 and 277 to 139 for PMEA and [13C]PMEA, respectively, by using negative electrospray ionization. Each transition was alternately monitored with a dwell time of 300 ms. Collision-activated dissociation gas was set at 10, curtain gas was set at 35 (arbitrary units). The TIS source temperature was maintained at 650°C and the IonSpray voltage was set at −3,500 V. Both the nebulizer (GS1) and TIS (GS2) were set at 50. Unit and low resolutions were applied for Q1 and Q3, respectively.

For remofovir, the calibration curve was linear over the concentration range of 4.93 to 1,260 ng/ml with a limit of quantitation at 4.93 ng/ml. Linear regression of the concentration data yielded a correlation coefficient (r) of >0.9992. The
LC-MS/MS method was accurate (bias, <5.5%) and reproducible (coefficient of variation, <10.8%).

For PMEA, the calibration curve was linear over the concentration range of 20.0 to 1,280 ng/ml with a limit of quantitation at 20.0 ng/ml. Linear regression of the concentration data yielded a correlation coefficient (r) of 0.9991. The LC-MS/MS method was accurate (bias, 0.9991) and reproducible (coefficient of variation, 9.3%).

Remofovir has been demonstrated to be stable at 4°C for at least 60 min in rat plasma. All precautions have been taken to ensure no in vitro degradation of remofovir to PMEA between blood collection and determination of concentrations.

HPLC procedure for studying the metabolic profiles of the drug in plasma and urine. For determining the metabolic profile, urine was analyzed directly without sample preparation. For plasma, the sample was first mixed with three equal volumes of a solution of acetone-water-trifluoroacetic acid (80/19.5/0.5, vol/vol/vol). The mixtures were centrifuged and the supernatant was collected for analysis. An HPLC device coupled with a radioactivity detector (β-Ram model 3; In/U Systems, Inc., Tampa, Fla.) was used for the analysis. The HPLC (Shimadzu model SCL 10VP) device was equipped with a Discovery C18 column (Supelco; 25 cm by 4.6 mm). The column was eluted with 100% of 0.1% acetic acid-water solution for 6 min at a flow rate of 1 ml/min. The organic phase (0.1% acetic acid in methanol) was increased from 0 to 60% under gradient conditions from 6 to 36 min. The organic mobile phase reached 100% in a second gradient program (4 min) and was maintained at this composition for 5 min. Identification of radioactive peaks in rat and monkey plasma and urine was based on the retention times of available standards and/or by a LC/MS technique. A Finnigan (San Jose, Calif.) LCO DekaXP mass spectrometer was used to obtain mass spectrometry fragmentation data for structure elucidation.

### Pharmacokinetic analysis
Concentrations of radioactivity, remofovir, and PMEA in plasma were used to determine pharmacokinetic parameters by using noncompartmental methods (Win Nonlin 1.5; Pharlmint Corp., Mountain View, Calif.). The maximum concentration (C\text{max}) and time of maximum concentration (T\text{max}) were observed values. The area under the concentration-time curve (AUC) to the last quantifiable sampling time, AUC(t), was computed using the linear trapezoidal rule. The area under the concentration-time curve to infinity, AUC(I), was calculated as the sum of AUC(t) and the quotient of the last measurable concentration, C(t), and the elimination rate constant (k\text{el}). k\text{el} was estimated as the negative slope of the regression of log concentration versus time. Half-life (t\text{1/2}) was calculated by dividing 0.693 by k\text{el}. The apparent total body clearance (CL) was calculated as the ratio of total body clearance to apparent volume of distribution (Vd) was calculated as the ratio of total body clearance to apparent volume of distribution (Vd).

### RESULTS
Concentrations of remofovir, PMEA, and radioactivity in rat plasma. Following i.v. administration of remofovir, plasma levels of remofovir decreased with time (Fig. 2) with an elimination t\text{1/2} of 0.7 h. The mean Vd was 5.99 liters/kg and the mean CL was 5.85 liters/h/kg. Following oral dosing of remo-
fovir, the drug was rapidly absorbed with a $T_{\text{max}}$ of 0.25 h and a $C_{\text{max}}$ of 0.105 mg/liter. Absolute bioavailability of remofovir was calculated to be 5.42% (Table 1).

After i.v. administration of remofovir, plasma levels of PMEA reached a $C_{\text{max}}$ at 0.5 h and then decreased with time (Fig. 2) with an elimination $t_{1/2}$ of 8.0 h. After oral dosing of remofovir, plasma levels of PMEA reached a maximum at 2 h with a $C_{\text{max}}$ of 0.268 mg/liter. Thereafter, plasma levels of PMEA decreased with time with an elimination $t_{1/2}$ of 7.3 h, which is longer than that for remofovir after either i.v. (0.7 h) or oral dosing (2.2 h) of remofovir. The PMEA AUC(I) was 3.03 mg·h/liter after i.v. dosing of remofovir and 1.73 mg·h/liter after oral dosing of remofovir (Table 1). Following i.v. administration of [14C]remofovir, plasma radioactivity declined with a $t_{1/2}$ of approximately 14.4 h. Absorption was estimated to be 29.7% (Table 1).

Concentrations of remofovir, PMEA, and radioactivity in monkey plasma. Following i.v. administration of remofovir, plasma levels of remofovir decreased with time (Fig. 2) with an elimination $t_{1/2}$ of 0.7 h. The mean Vd was 2.70 liters/kg and

FIG. 3. Metabolic profile of [14C]remofovir in rat and monkey plasma (30 min) following i.v. and oral (PO) dosing.
the mean CL was 2.60 liters/h/kg. After oral administration of remofovir, the drug was rapidly absorbed with a $T_{\text{max}}$ of 0.375 h and a $C_{\text{max}}$ of 1.80 mg/liter. Absolute bioavailability of remofovir was calculated to be 19.4% (Table 1).

Following i.v. administration of remofovir, plasma levels of PMEA decreased with time (Fig. 2) with an elimination $t_{1/2}$ of 4.6 h. Following oral dosing of remofovir, plasma PMEA levels reached a maximum at 0.5 h with a $C_{\text{max}}$ of 2.84 mg/liter. Thereafter, plasma PMEA levels declined with time with an elimination $t_{1/2}$ of 4.5 h. PMEA AUC(I) was 5.40 mg·h/liter after i.v. dosing and was 4.44 mg·h/liter after oral dosing of remofovir. Following i.v. administration of $[^{14}\text{C}]$remofovir, plasma radioactivity declined with a $t_{1/2}$ of 105 h. Absorption was estimated to be 65.6%.

Urinary and fecal excretion of radioactivity in rats and monkeys. In rats, 61.8% of the i.v. dose and 12.9% of the oral dose were excreted in urine (0 to 120 h). In feces, 37.5% of the i.v. dose and 86.7% of the oral dose were excreted (Table 1). These data demonstrate that biliary excretion played a significant role in the elimination of remofovir in rats (Table 1).

In monkeys, 43.3% of the i.v. dose and 34.9% of the oral dose were excreted in urine (0 to 168 h). In feces, 17.4% of the i.v. dose and 40.4% of the oral dose were excreted. These data
indicate that biliary excretion played a significant role in the elimination of remofovir in monkeys.

Metabolic profiles in plasma and urine from rats and monkeys. In rat and monkey plasma 0.5 h after i.v. administration of remofovir, remofovir caused the only major radioactive peaks, with PMEA, metabolite A, and metabolite B being the minor peak (Fig. 3). However, at 0.5 h after oral administration of remofovir, remofovir, PMEA, metabolite A, and metabolite B were all major radioactive peaks, indicating a significant first-pass effect in rats and monkeys.

In rat and monkey urine (0 to 24 h) after i.v. administration of remofovir, remofovir remained responsible for the major radioactive peak, while after oral administration, remofovir represented a minor radioactive peak with PMEA and metabolites A and B as major radioactive peaks (Fig. 4), confirming significant metabolism of remofovir and PMEA after oral dosing.

DISCUSSION

Following oral administration of remofovir to rats, mean \( T_{1/2} \) was 0.25 h for remofovir and 2 h for PMEA. Following oral administration of remofovir to monkeys, mean \( T_{1/2} \) was 0.375 h for remofovir and 0.5 h for PMEA. These data suggest that in both rats and monkeys, remofovir was rapidly absorbed and rapidly converted to PMEA.

In rats after i.v. dosing, the \( t_{1/2} \) for radioactivity (14.4 h) was longer than those for remofovir (0.7 h) and PMEA (8.0 h). In monkeys after i.v. dosing, the \( t_{1/2} \) for radioactivity (105 h) was much longer than those for remofovir (0.7 h) and PMEA (4.6 h). The longer \( t_{1/2} \) for radioactivity may be due to metabolite(s). However, we cannot contribute this long \( t_{1/2} \) to any specific metabolite, since we did not evaluate the pharmacokinetics of these metabolites in rats and monkeys.

Fecal excretion of radioactivity following i.v. dosing of \([^{14}C]\)remofovir accounted for 37.5% of the dose in rats and 17.4% of the dose in monkeys, indicating that biliary excretion played a more significant role in rats than in monkeys in the elimination of the drug. On the other hand, it has been reported (1) that PMEA is excreted into urine by a combination of glomerular filtration and active tubular secretion.

By comparing the plasma radioactivity AUC(I) obtained after oral dosing to that obtained after i.v. administration of \([^{14}C]\)remofovir, absorption of remofovir was estimated to be 29.7% in rats and 65.6% in monkeys. By comparing the plasma remofovir AUC(I) obtained after oral dosing to that obtained after i.v. administration of remofovir, bioavailability of remofovir was estimated to be 5.42% in rats and 19.4% in monkeys. Based on these data, the extraction ratio was estimated to be 0.82 in rats and 0.70 in monkeys, suggesting extensive metabolism of remofovir to PMEA and other metabolites in rats and monkeys. This is in good agreement with the observation that, following oral dosing, PMEA and other metabolites accounted for 23.3 and 78.6% of total radioactivity, respectively, in rat plasma, and 18.8 and 76.6% of total radioactivity, respectively, in monkey plasma (Table 1). Recently we have reported (C. Lin, A. Teng, L. T. Yeh, D. Vitarella, Z. Hong, J. Peterson, and M. Erion, Abstr. 39th Ann. Meet. Study Liver, abstr. 374, 2004) that in humans, following a single oral dose of 10, 30, or 60 mg of remofovir, the AUC(96 h) was 24.8, 110, and 180 ng · h/ml, respectively, for remofovir and 22.1, 145, and 278 ng · h/ml, respectively, for PMEA, indicating efficient conversion of remofovir to PMEA in humans.

It is important to note that clearance of remofovir following oral dosing was higher in rats (5.85 liters/h/kg) than in monkeys (2.60 liters/h/kg). Similarly, volume of distribution of remofovir in rats (5.99 liters/kg) was also higher than that in monkeys (2.70 liters/kg). The higher clearance of remofovir in rats than in monkeys following oral dosing is in good agreement with the findings that the ratio of plasma remofovir AUC(I) to plasma radioactivity AUC(I) was 1.93% in rats and 5.15% in monkeys (Table 1).

In rats, following oral administration of remofovir, the AUC(I) of PMEA (6.34 nM · h) was 10.9 times that of the AUC(I) of remofovir (0.582 nM · h), whereas following oral administration of remofovir in monkeys, the AUC(I) of PMEA (16.3 nM · h) was 3.47 times that of the AUC(I) of remofovir (4.68 nM · h). In humans, following oral dosing of 10 mg of remofovir (C. Lin et al., 39th EASL, abstr. 374), the AUC(I) of PMEA (0.148 nM · h) was 2.65 times that of the AUC(I) of remofovir (0.0558 nM · h), suggesting that monkeys, not rats, are more similar to humans in the metabolic conversion of remofovir to PMEA.

REFERENCES