# Metabolism and Pharmacokinetics of Novel Oral Prodrugs of 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) in Dogs

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*Received July 8, 1997; accepted September 15, 1997*

***Purpose.*** A series of prodrugs designed to enhance the oral bioavailability of the antiretroviral agent 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA; **1**) have been synthesized, including a bis-(acyloxymethyl) ester **2** and a series of bis-(alkoxycarbonyloxymethyl) esters **3–9**. The *in vitro* biological stability and *in vivo* pharmacokinetics of these prodrugs were evaluated to support selection of a prodrug candidate for clinical evaluation.

***Methods.*** The *in vitro* biological stability of the prodrugs was examined in dog tissues (intestinal homogenate, plasma and liver homogenate). The apparent half-lives were determined based on the disappearance of prodrug using reverse-phase HPLC with UV detection. Oral bioavailability of PMPA from each prodrug was determined in fasted beagle dogs. Concentrations of PMPA in plasma were determined by HPLC following fluorescence derivatization. Data for prodrugs were compared to historical data for intravenous PMPA.

***Results.*** All prodrugs were rapidly hydrolyzed in dog plasma and tissues ($t_{1/2} < 60$ min). In fasted beagle dogs, bis-[(pivaloyloxy)methyl] PMPA (bis-POM PMPA) **2** had the highest oral bioavailability as PMPA (37.8 ± 5.1%). The oral bioavailabilities of PMPA from bis-(alkoxycarbonyloxymethyl) esters ranged from 16.0% to 30.7% and PMPA was the major metabolite formed.

***Conclusions.*** There was a correlation between oral bioavailability and intestinal stability of bis-(alkoxycarbonyloxymethyl) ester prodrugs ($r^2 = 0.96$). Lipophilicity (log P) was not a good predictor of oral bioavailability. The most labile prodrugs in dog intestinal homogenates, bis-(n-butyloxycarbonyloxymethyl) PMPA **5** and bis-(neo-pentyloxycarbonyloxymethyl) PMPA **8** ($t_{1/2} < 5$ min) had the lowest oral bioavailabilities. Based on good oral bioavailability (30.1%), chemical and intestinal stability bis-(isopropyloxycarbonyloxymethyl) PMPA (bis-POC PMPA) **4** was selected as a candidate for clinical evaluation.

**KEY WORDS:** prodrug; 9-[(R)-2-(phosphonomethoxy)propyl]adenine; antiretroviral drug; oral bioavailability.

## INTRODUCTION

Acyclic phosphonate nucleotide analogs exhibit broad spectrum antiviral activity *in vivo* and their active metabolites have prolonged intracellular half-lives that allow for infrequent administration (1). The ionic nature of these agents limits their permeability across the human intestinal mucosa, which results in low oral bioavailability (2,3). Masking the negative charges of the phosphonate group with a biologically labile promoiety has been shown to enhance the oral bioavailability of several nucleotide phosphonates (4,5,6). For example, the oral bioavailability of the nucleotide analog 9-[2-(phosphonomethoxy)-ethyl]adenine (PMEA; adefovir) is low. The bis-[(pivaloyloxy)methyl] ester prodrug of PMEA (bis-POM PMEA; adefovir dipivoxil) was shown to significantly increase the oral bioavailability of PMEA in HIV infected patients (7).

PMPA (9-[(R)-2-(phosphonolmethoxy)propyl]adenine; GS-1278), is an acyclic nucleotide analog with potent and selective inhibitory activity *in vitro* against retroviruses (8). PMPA was effective in preventing simian immunodeficiency virus (SIV) infection in macaques, when the drug was administered up to 24 hours post infection (9). Intravaginal PMPA gel was also effective in preventing transmission of SIV (10). In a chronic SIV infection model, PMPA was effective for preventing vertical transmission of SIV from mother to newborn monkeys (11). Intravenous PMPA has been shown to reduce viral load by a median of 1.1 log units after only one week of dosing in HIV-infected patients (12). The oral bioavailability of PMPA is low in rats (6.0%), dogs (17.7%) and cynomolgus monkeys (5.3%) (13). In the present study, we evaluated the metabolism and bioavailability of a series of novel carbonate prodrugs of PMPA employing an oxycarbonyloxymethyl linker group. This linker group has been previously applied to various prodrug moieties (14,15). In addition, bis[(pivaloyloxy)methyl] PMPA (bis-POM PMPA) was also evaluated in the same *in vitro* and *in vivo* models for comparison.

## MATERIALS AND METHODS

### Materials

PMPA and its bis-(acyloxymethyl) or bis-(alkoxycarbonyloxymethyl) ester prodrugs were synthesized by Gilead Sciences as described elsewhere (16), the chemical structures are shown in Table 1. [8-$^{14}$C-adenine]-bis-(isopropyloxycarbonyloxymethyl) PMPA **4** was obtained as a solution in ethanol/water (50:50) from Moravek (Brea, CA). Chloroacetaldehyde and trifluoroacetic acid (TFA) were from Aldrich (Milwaukee, WI). All other chemicals used in this study were from standard commercial suppliers.

### *In Vitro* Biological Stability

Homogenates of dog intestine and liver were prepared following published protocols (18). Pooled dog plasma was obtained from Oread Laboratories, Inc. (Lawrence, KS). Metabolic stability studies in plasma and intestinal homogenate were performed at a final drug concentration of 10 μg/ml and in 90% biological fluid/2.5% DMSO. Each compound was incubated with liver S9 fraction in the presence of an NADPH generating system to facilitate cytochrome P450 mediated metabolism (17), to evaluate potential oxidative metabolism of phosphonate prodrugs (4). All prodrugs were soluble in the reaction mixtures. All tubes were preincubated without the drug in a shaker bath (New Brunswick Scientific, Edison, NJ) at 37°C and 100 oscillations/min for 5 minutes. The prodrug solution in DMSO was added to the incubation, mixed and maintained at 37°C and 100 oscillations/min. Aliquots (50 μl) were withdrawn at 1, 30 and 60 minutes and quenched with 100 μl of 0.1% TFA in acetonitrile. Quenched samples were centrifuged for 5

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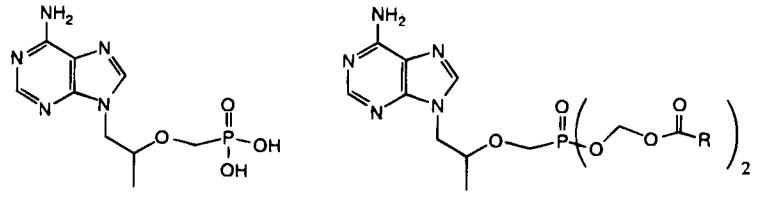
**Table 1.** Chemical Structures of PMPA Prodrugs



PMPA PMPA Prodrugs

| | Compound | R |
|---|---|---|
| **2** | Bis-(pivaloyloxymethyl) PMPA (bis-POM PMPA) | |
| Bis-(Alkoxycarbonyloxymethyl) esters | | |
| **3** | ethyl | |
| **4** | isopropyl | |
| **5** | n-butyl | |
| **6** | isobutyl | |
| **7** | t-butyl | |
| **8** | neo-pentyl | |
| **9** | 3-pentyl | |

minutes at 14,000 rpm in an Eppendorf Centrifuge 5402, and the supernatant was used directly for HPLC analysis. The HPLC system comprised a Model P4000 solvent delivery system with a Model AS3000 autoinjector and a Model UV1000 detector (Thermo Separation, San Jose, CA). The column was a Zorbax RX-C18 (5 μm, 250 × 4.6 mm) (MAC-MOD, Chadds Ford, PA) equipped with a Brownlee RP-18 Newguard guard column (7 μm, 15 × 3.2 mm) (Alltech, Deerfield, IL). The mobile phases were: A, 20 mM potassium phosphate buffer, pH 6.0; B, 65% acetonitrile in 20 mM potassium phosphate, pH 6.0. The gradient profile was a linear gradient to 100% B by 15.0 minutes, returning immediately to 100% A. The flow rate was 1.0 ml/min and the column temperature was maintained at 35°C by a column oven. Detection was by UV at 262 nm and the injection volume was 50 μl. Total cycle time between injections was 22 min. The observed rate constant for degradation ($k_{obs}$) was determined from the slope of a plot of the natural logarithm of the peak area of the prodrug versus time of incubation (min). The apparent half-life ($t_{1/2}$) was calculated as $0.693/k_{obs}$. For samples with less than 5% degradation after 60 min incubation, the prodrug was considered stable. For radiochromatography, detection was by a Radiomatic FLO-ONE/Beta liquid scintillation detector (Packard, Meriden, CT), using FLO-SCINT-A (Packard) as scintillation fluid.

### *In Vivo* Pharmacokinetics

The in-life phase of this study was conducted in accordance with the recommendations of the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication 86–23) and were approved by an Institutional Animal Care and Use Committee. Two groups of five adult male beagle dogs were used for the study. The mean body weight at the time of first dose was 9.1 ± 0.3 kg (range 8.7–9.5 kg). Dogs were fasted 12–18 hours prior to dosing and until 6 hours post-dose. Water was provided *ad lib.* Prodrugs were administered as solutions in 20% PEG 400/80% 50 mM citric acid (pH 2.2) (bis-POM PMPA 2 was formulated in 50 mM citric acid, pH 2.2). Chemical stabilities of these oral formulations were evaluated prior to dosing and minimal or no degradation was

observed. The oral formulations were each administered to five dogs by gavage, followed by two 10 ml water washes. At least one week washout period was allowed between administrations. The doses were 5.6–11 mg-equiv. of PMPA/kg for oral prodrugs. Blood samples (4.0 ml) were collected by direct jugular access from each animal into heparinized tubes at 0 (pre-dose), 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 hours post-dosing. Animals remained conscious throughout the sample collection period. Blood was processed immediately for plasma and was frozen and maintained at $\leq -70°C$ until analyzed.

### Determination of PMPA in Plasma

The concentrations of PMPA in plasma samples were determined by derivatizing PMPA with chloroacetaldehyde to yield a highly fluorescent $N^1$, $N^6$-ethenoadenine derivative (19). Plasma (200 μl) was mixed with internal standard (1 μg/ml of 9-[2-(phosphonomethoxy)ethyl]adenine; PMEA) and 400 μl of 0.1% TFA in acetonitrile to precipitate protein. Samples were evaporated to dryness under reduced pressure at room temperature, reconstituted in 200 μl derivatization cocktail (0.34% chloroacetaldehyde in 100 mM sodium acetate, pH 4.5), vortexed, and centrifuged. Supernatant was then transferred to a screw-capped eppendorf tube and incubated at 95°C for 40 minutes. Derivatized samples were evaporated to dryness and reconstituted in 200 μl mobile phase A (see below) for HPLC analysis. The HPLC system was the same as described above, except for a Model F2000 Fluorescence detector. The column was a Zorbax RX-C18 column (4.6 × 150 mm) equipped with a Brownlee RP-18 Newguard guard column (15 × 3.2 mm; Alltech, Deerfield, IL). The mobile phases used were: A, 5% acetonitrile in 20 mM potassium phosphate buffer with 5 mM tetrabutylammonium hydrogen phosphate (TBAHP), pH 6.0; B, 65% acetonitrile in 20 mM potassium phosphate buffer with 5 mM TBAHP, pH 6.0. The flow rate was 1.5 ml/min and the column temperature was maintained at 35°C by a column oven. The gradient profile was 100% A until 8 min, then a linear gradient to 100% B by 18 min, returning immediately to 100% A. Detection was by fluorescence with excitation at 236 nm and emission at 420 nm, and the injection volume was 20 μl. Total cycle time between injections was 26 min. Data was acquired and stored by a Peak Pro data acquisition system (Beckman, Palo Alto, CA).

### Determination of Prodrugs and Intermediates in Plasma

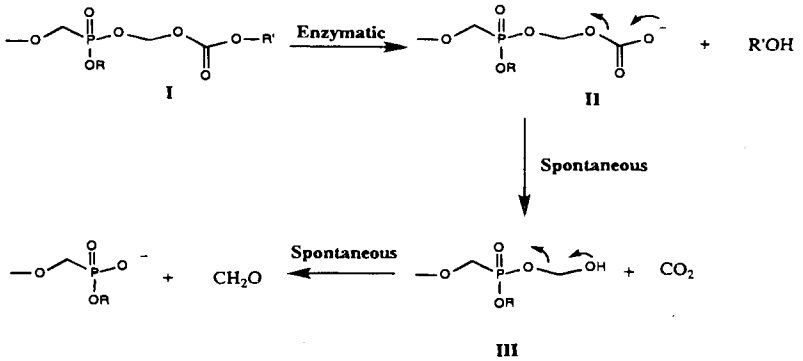
Concentrations of intact prodrug or intermediates in plasma were determined by a non-destructive HPLC-UV method. Plasma samples (50 μl) were mixed with 100 μl of 0.1% TFA in acetonitrile, centrifuged and supernatant was used for HPLC analysis. Samples were analyzed by the stability-indicating HPLC method described above.

### Pharmacokinetics and Statistical Analysis

Pharmacokinetic parameters for PMPA following oral administration of prodrugs were determined by non-compartmental methods using PCNONLIN (19). The bioavailable fraction of PMPA from an equimolar dose of prodrug (oral bioavailability; % F) was expressed as $((AUC\ (0\text{-}\infty)_{oral\ prodrug}/AUC\ (0\text{-}\infty)_{iv}\ PMPA) \times (Dose_{iv}/Dose_{oral} \times 100))$ (20). Pharmacokinetic parameters for oral prodrugs were compared by paired t-tests (for studies in the same set of animals) or unpaired t-tests (for studies in different sets of animals) (21). A P value of $\leq 0.05$ was considered significant.

## RESULTS AND DISCUSSION

Scheme 1 depicts the stepwise enzymatic and non-enzymatic reactions involved in the conversion of a phosphonate prodrug with an oxycarbonyloxymethyl linker group to the active drug. The conversion is initiated by esterase-mediated hydrolysis of the alkyloxycarbonyl group of **I** leading to the formation of the labile intermediate **II.** This intermediate is unstable and undergoes spontaneous decomposition to form the unstable intermediate **III** and carbon dioxide. Intermediate **III** subsequently decomposes to formaldehyde and the phosphonate monoesters. The monoester can undergo a similar pathway of degradation to yield the parent phosphonate. The degradation products for each mole of prodrug are therefore two moles each of alcohol, carbon dioxide, and formaldehyde. The chemical stability of these prodrugs has been examined (16) and the reactive intermediates depicted in scheme 1 (**II, III**) were not detected by HPLC analysis with UV detection (16). The half-life of the prodrug **7** (bis-(t-butyloxycarbonyloxymethyl) PMPA) at pH 7.4 is 15–35 fold shorter than the other prodrugs studied as shown in Table 2 (16). Prodrug **7** was 22 times more reactive at pH 7.4, 37°C than the corresponding bis-(isobutyloxycarbonyloxymethyl) PMPA **6**. The observed degradation rate constants for the other oxymethyloxycarbonyl containing prodrugs examined in the present study were similar to prodrug **6** (16). The chemical instability of prodrug **7** would likely present a major challenge for formulation development as well as increase the likelihood of hydrolysis in the lumen of the gastrointestinal tract.




**Scheme 1.**

**Table 2.** Chemical Stability, Partition Coefficients (Log P) and Molecular Weight of PMPA Prodrugs

| Prodrug | Half-life (hr) (pH7.4, 37°C)[a] | Log P (pH 6.5, 25°C)[a] | Molecular weight |
|---|---|---|---|
| **2** | 14 | 2.1 | 515 |
| **3** | 7.0 | 0.6 | 491 |
| **4** | 9.2 | 1.3 | 519 |
| **5** | 6.0 | 2.7 | 547 |
| **6** | 9.0 | 2.0 | 547 |
| **7** | 0.4 | 1.9 | 547 |
| **8** | 6.0 | >3.0 | 575 |
| **9** | 8.0 | >3.9 | 575 |

[a] Taken from ref. (16).

**Table 3.** *In Vitro* Stability of PMPA Prodrugs in Dog Tissues

| Compound | Half-life (min) | | |
|---|---|---|---|
| | Intestinal homogenate | Plasma | Liver homogenate |
| **2** | 10.4 | 35.5 | <5 |
| **3** | 23.3 | 16.6 | <5 |
| **4** | 52.6 | 20.5 | <5 |
| **5** | <5 | <5 | <5 |
| **6** | 15 | <5 | <5 |
| **7** | 26.6 | 21.2 | 14.9 |
| **8** | <5 | <5 | <5 |
| **9** | 30 | 15 | <5 |

*Note:* All studies were performed at 37°C.

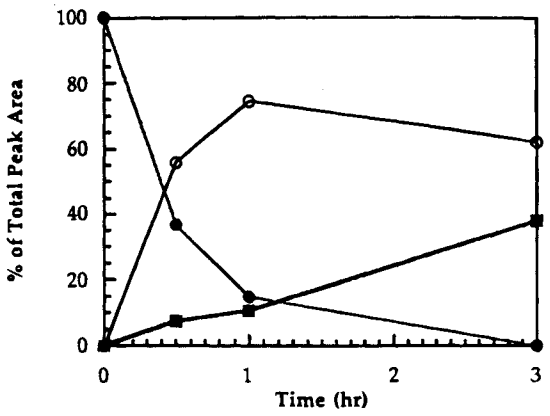



**Fig. 1.** *In vitro* stability profiles of $^{14}$C-bis-(isopropyloxycarbonyloxy methyl) PMPA **4** in dog intestinal homogenate following incubation at 37°C (●, Prodrug; ○, Monoester; ■, PMPA).

The relative hydrophobicities of the prodrugs were evaluated by measuring the octanol-water partition coefficient (log P) at pH 6.5 and 25°C as described in Table 2 (16). All the prodrugs examined were relatively lipophilic (log P > 0.5), suggesting that permeability would not be rate-limiting for oral absorption.

### *In Vitro* Stability in Dog Plasma and Tissues

The *in vitro* stability of bis-POM PMPA **2** and the bis-(alkoxycarbonyloxymethyl) ester prodrugs of PMPA **3–9** in dog plasma and tissue homogenates are summarized in Table 3. All prodrugs examined were relatively labile in dog plasma and tissues ($t_{1/2}$ < 5 min to 52.6 min). The major degradation products were the corresponding phosphonate monoesters, which degraded further on continued incubation to form PMPA as shown in Figure 1. The steric bulk of the alkyl group for the bis-(alkoxycarbonyloxymethyl) ester prodrugs was apparently the determining factor for *in vitro* biological stability. Stability in intestinal homogenate was greater for prodrugs with a secondary or tertiary alkyl substituent. The stability of the t-butyl derivative **7** in dog plasma and tissue homogenates was much greater than observed for the n-butyl analog **5**. A similar trend was observed for the neo-pentyl- and 3-pentyl- prodrugs **8** and **9**. All of the prodrugs examined were unstable in dog

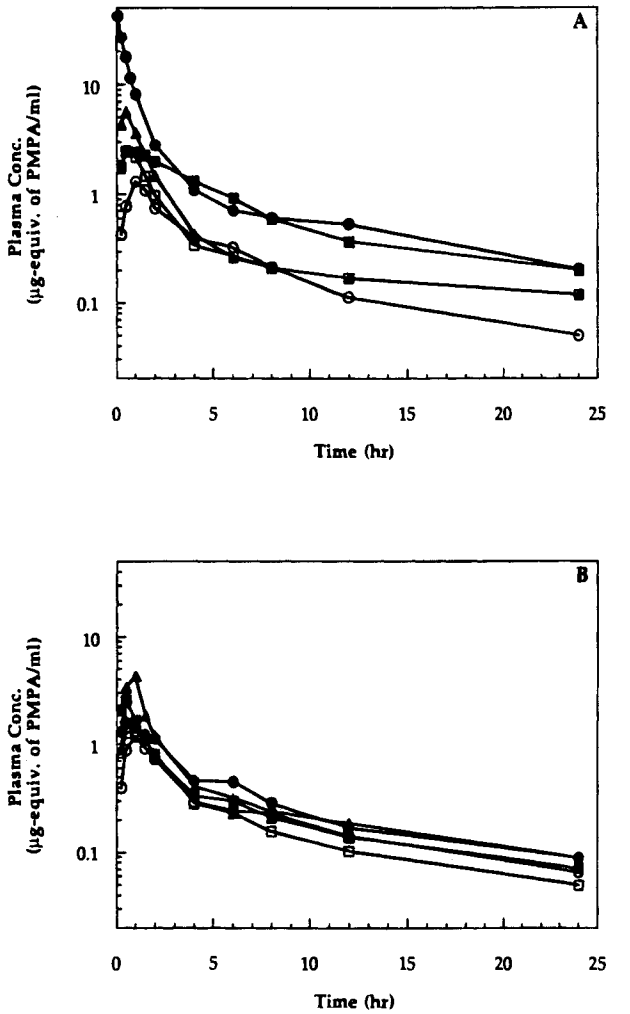



**Fig. 2.** Concentrations of PMPA in plasma following intravenous administration of PMPA or oral administration of PMPA or prodrugs to dogs. Data are mean of five animals. Concentrations are normalized to a dose of 10 mg equiv. of PMPA per kg. (A) ●, Intravenous PMPA **1**; ○, Oral PMPA **1**; ■, Oral Bis-POM PMPA 2; □, Oral Bis-POC PMPA **4**; ▲, Oral bis-(t-butyloxycarbonyloxymethyl) PMPA **7**. (B) ●, Oral bis-(ethyloxycarbonyloxymethyl) PMPA **3**; ○, Oral bis-(n-butyloxycarbonyloxymethyl) PMPA **5**; ■, Oral bis-(isobutyloxycarbonyloxymethyl) PMPA **6**; □, Oral bis-(neo-pentyloxycarbonyloxymethyl) PMPA **8**; ▲, Oral bis-(3-pentyloxycarbonyloxymethyl) PMPA **9**.

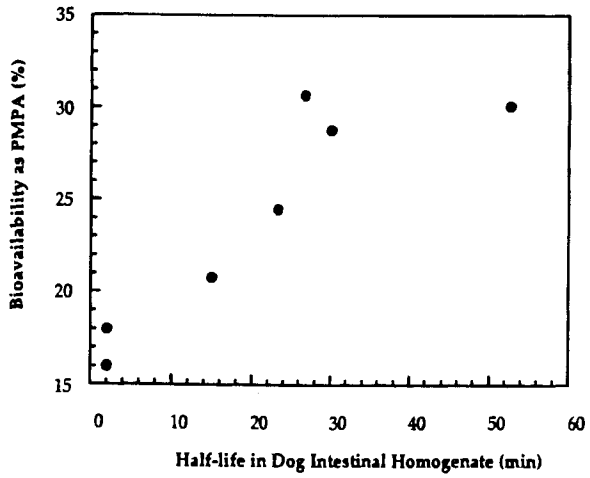



**Fig. 3.** Relationship between oral bioavailability of PMPA from bis-(alkoxycarbonyloxymethyl) ester prodrugs **3–9** and stability of the prodrugs in dog intestinal homogenate.

liver homogenate (Table 3). Preliminary data suggested that the degradation of bis-(isopropyloxycarbonyloxymethyl) PMPA **4** is not mediated by cytochrome P450 (not NADPH-dependent). Since the major degradation product for each prodrug is the corresponding monoester, a carboxylesterase appears to be required for the initial step in the hydrolysis of these prodrugs. While esterases are widely distributed in tissues and blood, the highest activities are found in liver (22). These data therefore support the observation that liver may be the primary site of metabolism for these prodrugs in dogs.

### Oral Bioavailability in Dogs

The plasma concentration versus time curve for PMPA in dogs following intravenous administration of PMPA **1,** is compared to that following oral administration of PMPA **1** and its prodrugs **2–9** in Figure 2. The oral bioavailabilities of PMPA from all eight PMPA prodrugs are summarized in Table 4. The same table shows the corresponding Cmax, Tmax values and terminal half-lives for PMPA in plasma. The oral bioavailability of PMPA from bis-(alkoxycarbonyloxymethyl) ester prodrugs of PMPA ranged from 16.0% to 30.7%. The oral bioavailability of PMPA from bis-POM PMPA **2** was 37.8±5.1% and was significantly higher than that observed for four of the bis-(alkoxycarbonyloxymethyl) ester prodrugs **3, 5, 6,** and **8** ($P<0.05$). All prodrugs examined showed similar Tmax values for PMPA (less than 2 hr) suggesting rapid conversion *in vivo.* PMPA was the major metabolite of bis-(alkoxycarbonyloxymethyl) ester prodrugs of PMPA **3–9** and bis-POM PMPA **2.** No intact prodrugs were detected in plasma. However, no steps were taken to prevent hydrolysis of intact prodrug during sample collection/preparation. Low concentrations of phosphonate monoester were detected following oral administration of bis-POM PMPA **2** (<0.5%), bis-(ethyloxycarbonyloxymethyl) PMPA **3** (2%), bis-(isopropyloxycarbonyloxymethyl) PMPA **4** (3.1%), and bis-(n-butyloxycarbonyloxymethyl) PMPA **5** (1%).

The relationship between intestinal stability of the bis-(alkoxycarbonyloxymethyl) ester prodrugs **3–9,** and the resulting oral bioavailability as PMPA is examined in Figure 3 ($r^2 = 0.96$ for prodrugs with primary alkyl substituent, n = 4). For the carbonyloxymethyl series, prodrugs with the greatest intestinal stabilities had the highest oral bioavailabilities. For example, bis-(isopropyloxycarbonyloxymethyl) PMPA **4** ($t_{1/2}$ =53 min) had a significantly higher oral bioavailability as PMPA than the prodrugs **5** or **8** ($t_{1/2} < 5$ min). These data suggested that absorption of these prodrugs is limited by hydrolysis in the lumen to the less permeable monoesters or PMPA. However, bis-POM PMPA **2** was well absorbed (oral bioavailability: 37.8%) despite a short half-life in dog intestinal homogenate ($t_{1/2}$ = 10.4 min). In the later case, lipophilicity as opposed to intestinal lumen stability may be the critical parameter governing bioavailability. Liver is most likely the site of metabolism for absorbed prodrugs based on results from *in vitro* stability study (Table 3). The relative lipophilicities of the prodrugs were evaluated by measuring the octanol-water partition coefficient (log P) at pH 6.5 and 25°C as shown in Table 2 (16). The

**Table 4.** Pharmacokinetics of PMPA Prodrugs Following Oral Administration to Beagle Dogs

| Compound | Dose (mg-equiv. of PMPA/kg) | AUC (0-∞) (μg.hr/ml) | PMPA Cmax[a] (μg/ml) | PMPA Tmax (hr) | % F as PMPA | Terminal Half-life (hr) |
|---|---|---|---|---|---|---|
| **2** | 7.5 | 11.1±1.5 | 2.7±0.6 | 0.8±0.7 | 37.8±5.1 | 14.8±2.9 |
| **3** | 11.2 | 10.7±1.3 | 1.6±0.3 | 1.1±0.8 | 24.5±2.9 | 11.2±5.5 |
| **4** | 9.5 | 10.5±4.9 | 2.6±0.6 | 0.7±0.3 | 30.1±12.4 | 14.3±3.3 |
| **5** | 8.0 | 6.3±1.4 | 1.1±0.3 | 1.0±0.4 | 18.0±4.0 | 11.7±7.4 |
| **6** | 7.2 | 5.9±2.1 | 2.5±0.5 | 0.5±0.0 | 20.8±7.5 | 9.1±2.0 |
| **7** | 6.5 | 7.7±1.4 | 5.6±1.5 | 0.4±0.1 | 30.7±5.7 | 15.6±7.5 |
| **8** | 7.7 | 4.8±1.7 | 1.4±0.2 | 0.8±0.3 | 16.0±5.6 | 8.4±3.0 |
| **9** | 6.2 | 7.0±1.9 | 5.1±0.5 | 0.8±0.3 | 28.8±7.6 | 10.1±2.3 |
| Intraveneous PMPA[b] **1** | 9.4 | 36.7±7.9 | 33.1±6.4 | — | 100 | 9.5±1.2 |

*Note:* Data are mean ± SD for 5 animals.

[a] Cmax values were normalized to a dose of 10 mg-equiv. of PMPA/kg dose level.

[b] Data for intravenous PMPA is unpublished. The Cmax value for intravenous PMPA is the extrapolated Co value (initial plasma concentration).

log P values of bis-POM PMPA **2** is higher than that of a carbonyloxymethyl prodrug with similar molecular weight (bis-POC PMPA **4**) (log P: 2.1 and 1.3 for **2** and **4**, respectively).

In summary, the bis-(alkoxycarbonyloxymethyl) ester prodrugs of PMPA successfully delivered PMPA to the systemic circulation. The oral bioavailability of PMPA from the bis-(alkoxycarbonyloxymethyl) ester prodrugs (range: 16.0 to 30.7%) appears to correlate with their *in vitro* stability in an intestinal homogenate. Based on its good oral bioavailability (30.1%), chemical and intestinal stability, bis-(isopropyloxycarbonyloxymethyl) PMPA (bis-POC PMPA) **4** was selected for further development and is currently being evaluated in phase I/II clinical trials in patients with HIV-infection.

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