A Peptide Prodrug Approach for Improving Bisphosphonate Oral Absorption

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A Peptide Prodrug Approach for Improving Bisphosphonate Oral Absorption

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This work was aimed at improving the absorption of bisphosphonates by targeting carrier systems in the intestine and the intestinal peptide carrier system (hPEPT1), in particular. ^{14}C-Labeled pamidronate and alendronate as well as radiolabeled and "cold" peptidyl-bisphosphonates, Pro-[^{3}H]Phe-[^{14}C]pamidronate, and Pro-[^{3}H]Phe-[^{14}C]alendronate were synthesized. In situ single-pass perfusion studies revealed competitive inhibition of transport by Pro-Phe, suggesting peptide carrier-mediated transport. Prodrug transport in the Caco-2 cell line was significantly better than that of the parent drugs, and the prodrugs exhibited high affinity to the intestinal tissue. Oral administration of the dipeptidyl prodrugs resulted in a 3-fold increase in drug absorption following oral administration in rats, and the bioavailability of Pro-Phe-alendronate was 3.3 (F_{TIBIA}) and 1.9 (F_{URINE}) times higher than that of the parent drug. The results indicate that the oral absorption of bisphosphonates can be improved by peptidyl prodrugs via the hPEPT1; however, other transporters may also be involved.

Introduction

A number of geminal bisphosphonates have been approved for clinical use in Paget's disease, tumor osteolysis, and hypercalcemia of malignancy, and alendronate has been recently approved for osteoporosis[1−5] (see Figure 1). The major disadvantage of the clinically utilized bisphosphonates is their poor absorption from the GI tract. Less than 1% of the oral dose is absorbed, and absorption is suppressed in the presence of food.[6−10] Furthermore, the absorption is variable between individuals and within the same individual, making accurate oral dosage of these compounds difficult. The poor absorption of bisphosphonates, tetraacids almost completely ionized at physiological pH, is attributed to their high polarity which prevents transcellular transport across the epithelial barriers.[11] In addition, the very poor solubility of the metal salts/complexes formed from bisphosphonates in the GI tract is another possible reason for poor absorption.[12] In the treatment of osteoporosis the variable and less than 1% absorption of the bisphosphonates is clinically effective, since it is administered chronically and most of the absorbed drug strongly binds to bone. However, to obtain an effective antiresorptive effect in tumor osteolysis, a relatively high dose of bisphosphonates must be given parenterally.[13] The preferred mode of administration is by iv infusion, since im and sc injections are associated with local necrosis. Thus, if increased absorption could be attained, oral administration would be a viable delivery system in situations where higher doses are required. In addition bisphosphonate therapy in osteoporosis would improve significantly.

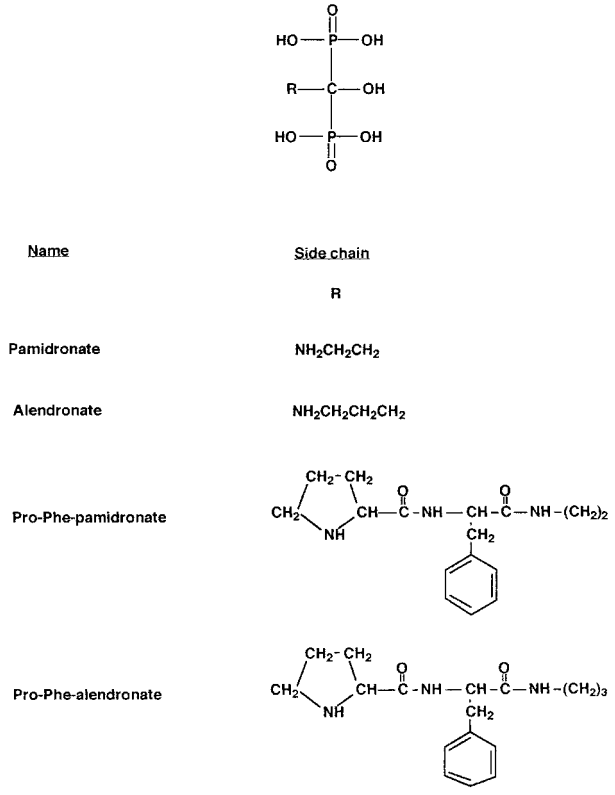

Figure 1. Structures of pamidronate, alendronate, Pro-Phe-pamidronate, and Pro-Phe-alendronate.

A relatively novel method to increase membrane permeability is by utilizing the carrier systems of the brush-border membrane of intestinal mucosa.[14] The

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active transporter, hPEPT1, is responsible for di/tripeptide absorption and for absorption of β-lactam antibiotics,[15] renin inhibitors,[16] and angiotensin-converting enzyme (ACE) inhibitors.[17,18] It was found that dipeptide prodrugs Phe-α-methyldopa, α−methyldopa-Phe, and α-methyldopa-Pro demonstrate more than 10 times higher intestinal permeability than the parent drug L-α-methyldopa, a polar amino acid, that is poorly transported by the amino acid transporter.[19] There are no reports on orally absorbable bisphosphonates or on application of the peptide prodrug approach to enhance GI absorption of these drugs. In this work bisphosphonates (pamidronate and alendronate, the most active bisphosphonates approved for clinical use) were converted into the peptidyl prodrugs prolyl-phenylalanyl-pamidronate (Pro-Phe-pamidronate) and prolyl-phenylalanyl-alendronate (Pro-Phe-alendronate). This work was aimed at improving the absorption of bisphosphonates by targeting carrier systems in the intestine and the intestinal peptide carrier system, in particular. It was hypothesized that the prodrug would be actively transported into the cytosol and would subsequently be hydrolyzed by cytosolic enzymes to the parent drug.

Results

1. Synthesis of Bisphosphonates and Prodrugs. Pamidronate-*1-^{14}C* and alendronate-*1-^{14}C* were synthesized from 3-aminopropanoic-*1-^{14}C* acid and 4-aminobutyric-*1-^{14}C* acid, respectively, by modification of a procedure involving the reaction of carboxylic acids with phosphorus trichloride, in the presence of pyridine hydrochloride.[20]

N-t-Boc-L-prolyl-L-phenylalanine was activated by conversion to *N*-hydroxysuccinimide ester, which in turn was condensed with the aminoalkyl-bisphosphonates in the presence of 3.5 equiv of diisopropylethylamine. This reaction was carried out in aqueous 2-propanol. In this solvent mixture it was possible to obtain a homogeneous reaction mixture containing both the ionic bisphosphonate salt and the "organic" peptide derivative. It was possible to monitor the progress of the reaction by ^{31}P NMR spectroscopy, due to the small shift of the ^{31}P signal caused by the attachment of the peptide chain. The peptidyl-bisphosphonates were isolated from the reaction mixtures by precipitation with benzathine and recovery using a cation exchange column. Pro-Phe-pamidronate was characterized by elemental analysis, ^{1}H and ^{31}P NMR spectroscopy, and FAB mass spectrometry. Pro-Phe-alendronate was characterized by elemental analysis, ^{1}H and ^{31}P NMR spectroscopy, and FAB mass spectrometry. The labeled bisphosphonates and peptidyl-bisphosphonates were identified only by ^{1}H and ^{31}P NMR spectroscopy.

2. iv Administration. The distribution of Pro-Phe-alendronate and alendronate in calcified and noncalcified tissues of rats is shown in Table 1. Following iv administration of 2 mg/kg Pro-[^{3}H]Phe-[^{14}C]alendronte (equivalent to 1 mg/kg alendronate) the amount of ^{14}C (bisphosphonate moiety) in urine was not significantly different than that found following alendronate administration (Table 1). Significantly higher amounts of alendronate were disposed in soft tissues of liver and kidney following prodrug administration (5.6 and 2.5 times higher in the liver and kidney, prodrug and alendronate, respectively) and significantly lower amounts of alendronate were disposed in the tibia following prodrug administration (1.2 times lower). Analysis of the ^{3}H isotope in urine and bone specimens resulted in similar results (data not shown).

Table 1. Distribution of [^{14}C]Alendronate and Pro-[^{3}H]Phe-[^{14}C]alendronate in Rat ($n = 6$) Tissues after iv Dose of 1 mg/kg Alendronate

	distribution (% dpm or % dpm/g tissue)	
tissue	[^{14}C]alendronate	Pro-Phe-[^{14}C]alendronate
urine	19.4 ± 6.9	23.9 ± 5.01
tibia	5.2 ± 0.4	4.2 ± 0.45*
femur	3.8 ± 0.6	3.2 ± 0.28
kidney	0.28 ± 0.1	1.59 ± 0.38*
liver	0.048 ± 0.02	0.119 ± 0.02*
feces	0.88 ± 0.3	2.2 ± 1.41

*Differences were termed statistically significant by two-tailed nonparametric Mann−Whitney U-test ($n = 6$, mean ± SD, $p < 0.05$).

3. Oral Absorption and Bioavailability.

3.1. Pro-Phe-pamidronate: Following oral administration of 20 mg/kg Pro-[^{3}H]Phe-[^{14}C]pamidronate (equivalent to 10 mg/kg pamidronate) the urine amount of ^{14}C (bisphosphonate moiety) was 3 times higher than that found following pamidronate administration (0.64 and 0.21% dpm, respectively, Figure 2). The levels of ^{14}C in the tibia, resulting from the administration of prodrug, were 4.8 times higher than those found following parent drug administration (Figure 2). The detection of the prodrug labeled with the two isotopes in the urine revealed 2.5 times higher amounts of the peptide moiety (^{3}H counts) than that of the bisphosphonate (^{14}C counts, Figure 3). In soft tissues, especially in the liver, high concentrations of the peptide moiety were detected, with similar concentrations in the tibia (Figure 3).

3.2. Pro-Phe-alendronate: Following oral administration of 20 mg/kg Pro-[^{3}H]Phe-[^{14}C]alendronte (equivalent to 10 mg/kg alendronate) the urine amount of ^{14}C (bisphosphonate moiety) was 2 times higher than that found following 10 mg/kg alendronate administration (0.74 and 0.32% dpm, respectively, Figure 2). Most of the prodrug was found in the feces (79.4%, Figure 2). Following prodrug administration, the concentration of ^{14}C in the tibia was 2.7 times higher than that found following parent drug administration (Figure 2). The analyses of the different isotopes (^{3}H and ^{14}C) in the urine revealed higher levels of the peptide moiety (^{3}H counts) than the bisphosphonate moiety (^{14}C counts), (3.83 and 0.74% dpm, respectively, Figure 3). The levels of ^{3}H found in the feces were lower than those of ^{14}C (Figure 3). The levels of the peptide moiety (^{3}H) in the soft tissues were 73 times higher than those of the bisphosphonate moiety (^{14}C) (e.g. in the liver, 0.22 and 0.003% dpm/g, respectively) with similar levels in the tibia (Figure 3).

3.3. Bioavailability: The bioavailability of Pro-Phe-alendronate calculated according to drug accumulation in bone and in urine, F_{TIBIA} and F_{URINE}, were 1.74% and 3.10%, respectively (Table 2). The bioavailabilities of the parent drug, F_{TIBIA} and F_{URINE}, were, respectively, 3.3 and 1.9 times lower than that of the prodrug (Table 2). Similar results were found for pamidronate (Table 2).

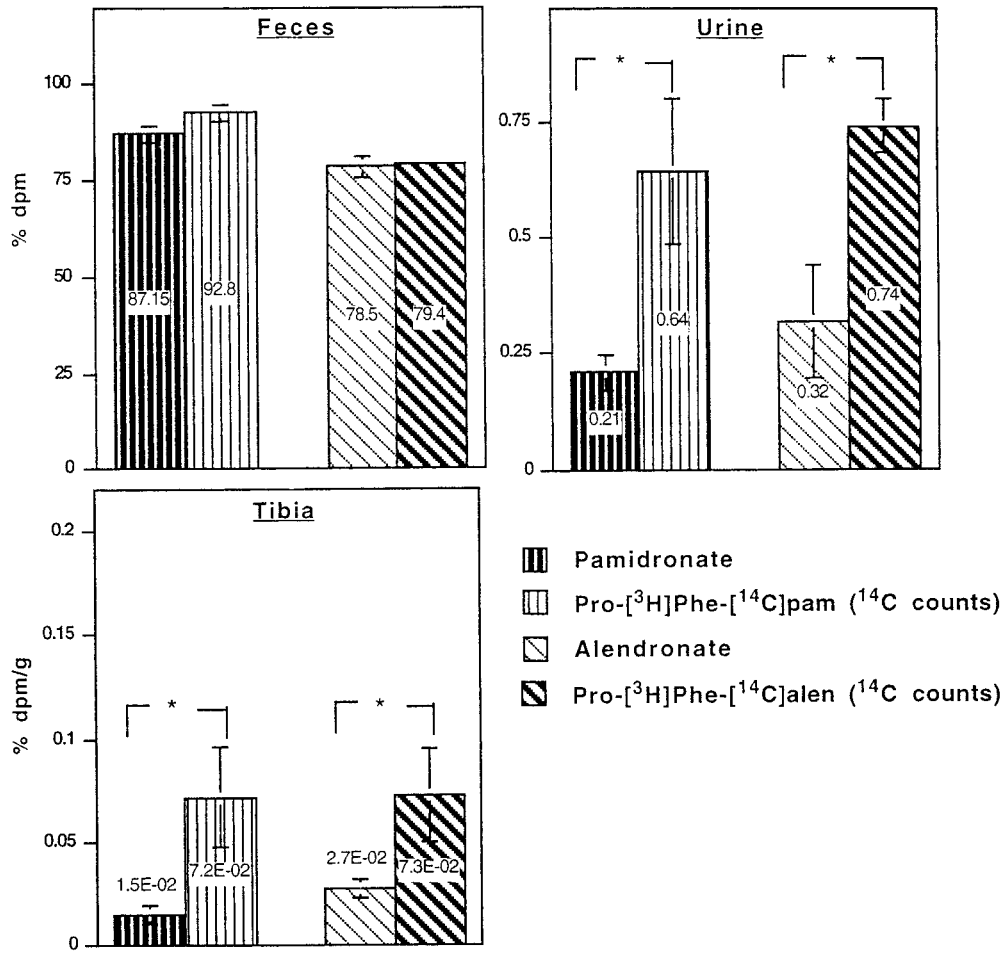

Figure 2. Disposition of Pro-[^{3}H]Phe-[^{14}C]pamidronate, [^{14}C]pamidronate, Pro-[^{3}H]Phe-[^{14}C]alendronate, and [^{14}C]alendronate (^{14}C counts) in rat urine, feces, and tibia 24 h after po administration. The tested compounds (10 and 20 mg/kg pamidronate/alendronate and Pro-Phe-pamidronate/alendronate, respectively) were administered po to rats housed in metabolic cages, and the amounts of ^{14}C were determined 24 h after administration. Data are expressed as % dpm in feces and urine and as % dpm/g tibia weight. *Differences were termed statistically significant by the Mann−Whitney one-tailed test (mean ± SE, $n = 6$, $p < 0.05$).

4. Mechanism of Prodrug Absorption.

4.1. Gastric absorption: To determine the site of prodrug absorption and the mechanism, prodrug absorption was studied in pylorus-ligated rats. The amount of pamidronate and Pro-Phe-pamidronate detected in the tibia after peroral administration in pylorus-ligated rats is presented in Figure 4. The levels of ^{14}C in bone following oral administration of pamidronate and Pro-Phe-pamidronate in pylorus-ligated rats were similar in both the femur (9.36×10^{-3} and 1.17×10^{-2}% dpm/g, respectively, data not shown) and the tibia (9.37×10^{-3} and 1.08×10^{-2}% dpm/g, respectively). However, a markedly better absorption of the prodrug was observed in control animals (approximately 3.8-fold in the femur and 4.7-fold in the tibia).

4.2. In situ single-pass perfusion: Figure 5 shows the effective permeability coefficients of the parent drugs, the dipeptide, and the prodrugs. The permeability of Pro-Phe-pamidronate was studied in the absence and presence of a competitive inhibitor (Pro-Phe). Compared to the parent drugs, the effective permeability coefficients of Pro-Phe-pamidronate and Pro-Phe-alendronate were 7.9- and 6.9-fold higher, respectively. No difference was found between the values of P_{eff} calculated on the basis of ^{14}C or ^{3}H counts (for Pro-Phe-pamidronate 1.79×10^{-5} and 1.90×10^{-5} cm/s, respectively; for Pro-Phe alendronate 1.10×10^{-5} and 2.3×10^{-5} cm/s, respectively). The transport rate of Pro-Phe-pamidronate was suppressed 2-fold by the competitive inhibitor Pro-Phe.

4.3. Uptake of Pro-Phe-alendronate in the intestinal wall: Figure 6 shows the time-dependent uptake of the prodrug in comparison to alendronate in rat intestine wall tissue after oral administration. The uptake vs time was in a bell shape, and the amount of prodrug in the intestinal wall was higher at all time points than that of the parent drug (^{14}C and ^{3}H counts). These results suggest that the prodrug has higher affinity to the intestinal tissue than the parent drug. This may suggest a carrier/active transport, although other reasons such as hydrophobic bonding are possible.

5. Transport in the Caco-2 Cell Line. Table 3 shows the apical (ap) to basolateral (bl) and bl to ap fluxes of mannitol, Pro-Phe, alendronate, pamidronate, and their dipeptidyl prodrugs across Caco-2 cell monolayers. The prodrugs Pro-Phe-pamidronate and Pro-Phe-alendronate (bisphosphonate moiety, ^{14}C) from the ap to bl direction were transported significantly better than the parent drugs (2.2 and 3.8 times higher, respectively).

Analyses of the different isotopes (^{3}H and ^{14}C) revealed that the transport of the dipeptidyl moiety (Pro-

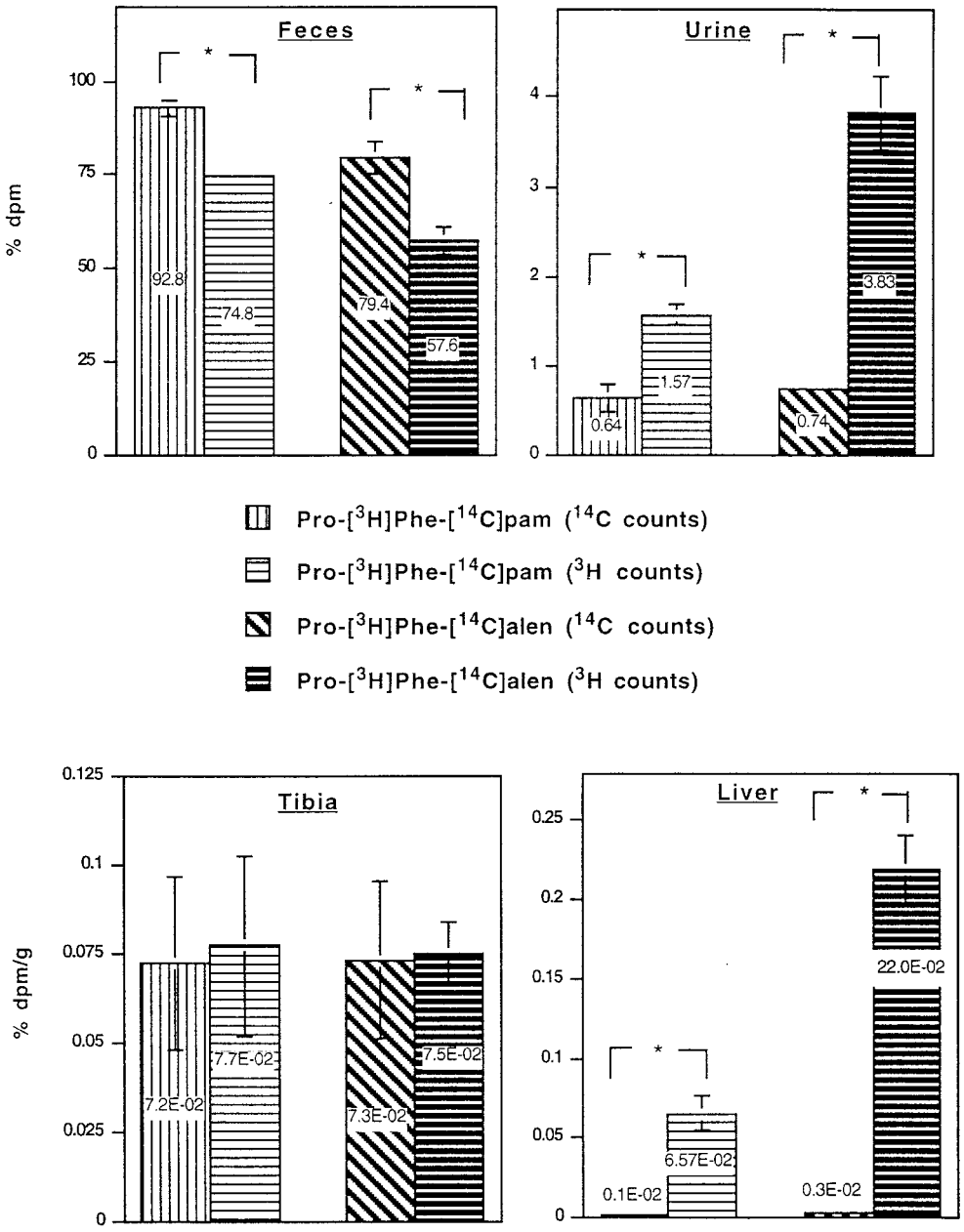

Figure 3. Disposition of Pro-[^{3}H]Phe-[^{14}C]pamidronate and Pro-[^{3}H]Phe-[^{14}C]alendronate (^{14}C and ^{3}H counts) in rat urine, feces, tibia, and liver organs 24 h after po administration. The tested compounds (10 and 20 mg/kg pamidronate/alendronate and Pro-Phe-pamidronate/alendronate, respectively) were administered po to rats housed in metabolic cages, and the amounts of ^{14}C and ^{3}H were determined 24 h after administration by means of a sample oxidizer. Data are presented as % dpm in feces and urine and as % dpm/g tibia or liver weight. *Differences were termed statistically significant by the Mann−Whitney one-tailed test (mean ± SE, $n = 6$, $p < 0.05$).

Table 2. Prodrug Oral Bioavailability[a]

	Pro-Phe-[^{14}C]-alendronate	alendronate	Pro-Phe-[^{14}C]-pamidronate	pamidronate
F_{URINE}	3.10*	1.65	3.17*	1.74
F_{TIBIA}	1.74*	0.52	1.65*	0.44

[a] Bioavailability values of the prodrugs were estimated by the ratios of $^{14}C_{po}/^{14}C_{iv}$ accumulated in bone and urine 24 h following administration (see Experimental Section). *Significantly different from the respective parent drug by one-tailed nonparametric Mann−Whitney U-test ($n = 6$, mean ± SD, $p < 0.05$).

Phe) was significantly higher from the ap to bl direction in comparison to the bl to ap direction, indicating active transport. The transport of the bisphosphonate moieties (^{14}C counts of pamidronate and alendronate) of both prodrugs was insignificantly higher from the ap to bl direction in comparison to the bl to ap direction. These results may suggest active/carrier-mediated transport.

6. Bioconversion. The most significant hydrolysis of Pro-Phe-pamidronate was found in the intestinal mucosal homogenate of the jejunum segment (36%, Table 4). When the prodrug concentration was increased from 0.25 to 1 mM, the hydrolysis in the same concentration of jejunum segment was decreased to 12%. No significant hydrolysis was detected in artificial gastric juice or other segments of the GI tract (Table 4).

7. Biological Activity.

7.1. Anticalcification effects: Table 5 presents the in vitro effects of pamidronate and Pro-Phe-pamidronate on the extent of HAP precipitation. Both drugs significantly inhibited HAP formation at both concentrations

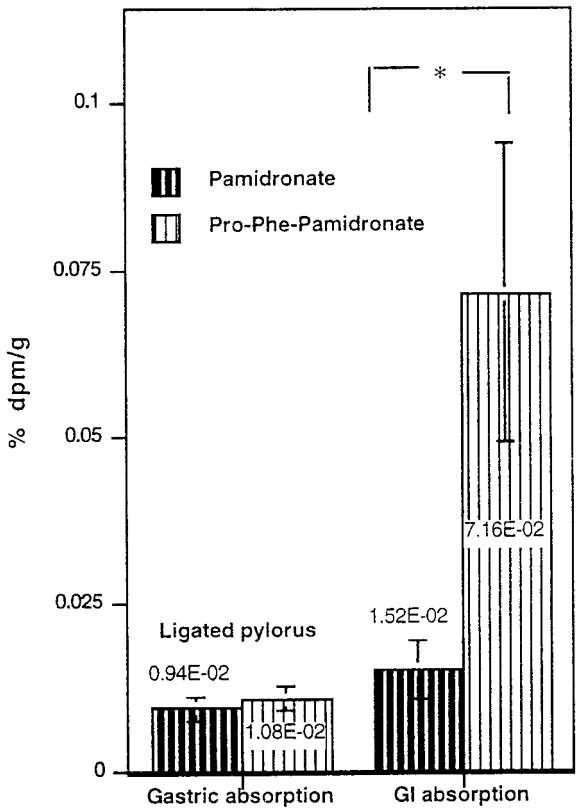

Figure 4. Disposition of Pro-[^{3}H]Phe-[^{14}C]pamidronate in rat tibia 2 h after po administration in pylorus-ligated rats and 24 h after po administration in control animals. [^{14}C]Pamidronate and Pro-[^{3}H]Phe-[^{14}C]pamidronate (10 mg/kg pamidronate) were administered po to pylorus-ligated rats to avoid intestinal absorption (active carrier-associated absorption). The amounts of ^{14}C and ^{3}H were determined 2 h after po administration. Data are presented as % dpm (^{14}C counts)/g tibia weight. *Differences were termed statistically significant by the Mann−Whitney one-tailed test (mean ± SE, $n = 6$, $p < 0.05$).

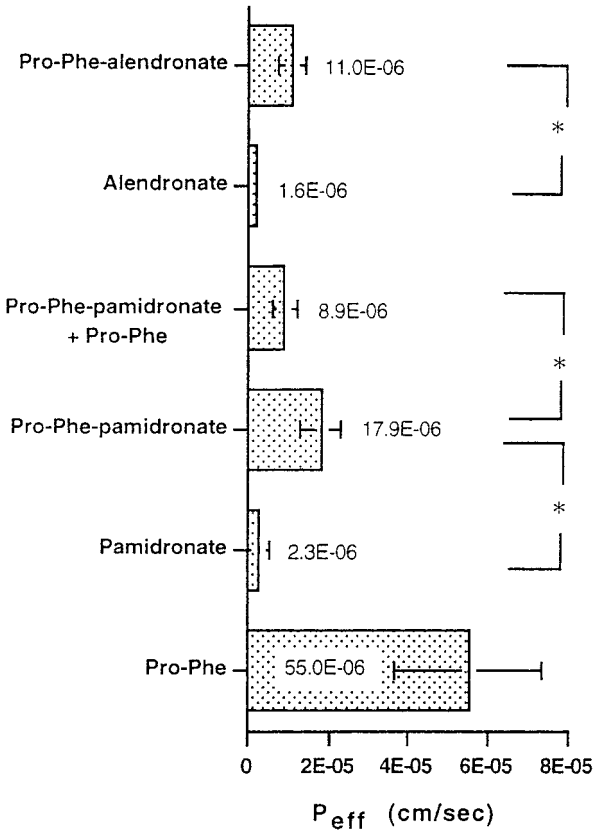

Figure 5. Effective permeability coefficients of rat jejunum for the parent drugs (pamidronate and alendronate), the dipeptide (Pro-Phe), and the prodrugs (Pro-Phe-pamidronate/alendronate). The jejunal segment was perfused by single pass in situ (rate of 0.19 mL/min and 0.09 mM tested compound). The permeability of Pro-Phe-pamidronate was tested in the absence and presence of a competitive inhibitor (4 mM Pro-Phe). *Differences were termed statistically significant by the Mann−Whitney one-tailed test (mean ± SE, $4 \leq n \leq 8$, $p < 0.05$), except for Pro-Phe-pamidronate vs Pro-Phe-pamidronate + Pro-Phe, $p = 0.058$.

(0.1 and 1 mM). The in vitro anticalcification activity of pamidronate and Pro-Phe-pamidronate was found to decrease at the higher concentration. This apparent inactivity was due to the low solubility of the calcium salts/complexes of the former two compounds, rather than increased HAP formation.[21]

Figure 7 depicts the inhibition of bioprosthetic heart valve tissue calcification by Pro-Phe-pamidronate, Pro-Phe-alendronate, pamidronate, and alendronate. The prodrugs and bisphosphonates significantly inhibited tissue calcification in the following order: alendronate = pamidronate > Pro-Phe-alendronate = Pro-Phe-pamidronate. The anticalcification effect of the various compounds was not associated with adverse effects on somatic growth as evidenced by the normal weight gain of the treated animals.

7.2. Effects on HAP dissolution: Table 5 shows the effect of pamidronate and Pro-Phe-pamidronate on the dissolution of HAP tablets at pH 5. Pro-Phe-pamidronate was found to be as effective as pamidronate in inhibiting HAP dissolution in this in vitro model.

Discussion

There are two common ways to improve oral absorption of highly hydrophilic polar drugs: (1) the use of absorption enhancers and (2) the design of lipophilic prodrugs. Unfortunately, the former approach has led to damage of the epithelial cells and/or has resulted in only very limited success when applied to bisphosphonates.[12,22] A recent paper describes a prodrug of clodronate (clodronic acid dianydrides), but no absorption/permeation studies have been reported.[23] Because of its involvement in the oral absorption of β-lactam antibiotics, renin inhibitors, and ACE inhibitors, the oligopeptide transporter has received much attention as a drug delivery system. Hu et al.[19] studied the possibility to improve the absorption of L-α-methyldopa by synthesizing peptide analogues that target to the peptide carrier system. The permeability of the dipeptide prodrugs Phe-α-methyldopa, α-methyldopa-Phe, and α-methyldopa-Pro increases significantly in comparison to the parent compound. Recently similar results were found for valcyclovir in comparison to acyclovir.[24] In this study we applied the same approach to bisphosphonates. The brush-border membrane of intestinal mucosa contains an active transporter for di- and tripeptides (hPEPT1). In this work, the bisphosphonates were coupled to the dipeptide, proline-phenylalanine, which is known to be actively transported by the peptide carrier.[25] Earlier studies in animals performed by Lin et al.[8,26] have demonstrated that following systemic administration,

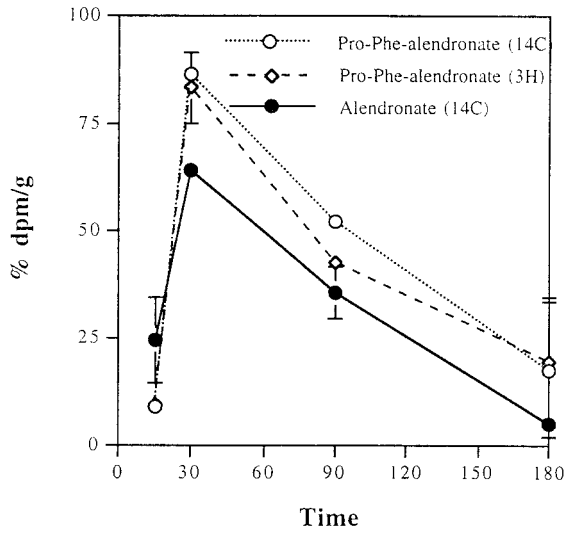

Figure 6. Time-dependent uptake of Pro-Phe-alendronate and alendronate by the intestine following po administration. The tested compounds (10 and 20 mg/kg alendronate and Pro-Phe-alendronate, respectively) were administered po to rats housed in metabolic cages, and the amounts of ^{14}C and ^{3}H were determined 0.25, 0.5, 1.5, and 3 h after administration by means of a sample oxidizer. Data are presented as % dpm/g intestine (duodenum) weight (mean ± SE, n = 2/time point).

Table 3. Apical to Basolateral and Basolateral to Apical Fluxes of Mannitol, Proline-Phenylalanine, Alendronate, Pamidronate, and Their Dipeptidyl Prodrugs (Pro-[^{3}H]Phe-[^{14}C]alendronate and Pro-[^{3}H]Phe-[^{14}C]pamidronate) across Caco-2 Cell Monolayers at 37 °C[a]

compound	permeability $\times 10^{-7}$ (cm/s) ap−bl	bl−ap
mannitol	2.6 ± 1.0	2.2 ± 1.0
Pro-Phe	22.9 ± 8.5	11.4 ± 0.6*
alendronate	0.6 ± 0.1	0.6 ± 0.1
Pro-Phe-alendronate (Pro-Phe)	10.3 ± 2.0**	5.5 ± 1.2*
Pro-Phe-alendronate (alendronate)	2.3 ± 2.4**	1.6 ± 0.7
pamidronate	0.5 ± 0.2	0.4 ± 0.2
Pro-Phe-pamidronate (Pro-Phe)	5.0 ± 0.2**	4.1 ± 0.2*
Pro-Phe-pamidronate (pamidronate)	1.1 ± 0.5**	0.9 ± 0.06

[a] Differences were termed statistically significant by the Mann−Whitney one-tailed test (mean ± SD, $n > 6$, $p < 0.05$), except for Pro-Phe-alendronate (^{14}C counts, alendronate) vs alendronate, $p = 0.0564$. *Significantly different in comparison to ap−bl ($p < 0.05$). **Significantly different in comparison to parent bisphosphonate ($p < 0.05$).

Table 4. Enzymatic Hydrolysis of Pro-Phe-pamidronate in Rat Jejunum Homogenate and Artificial Gastric Juice[a]

GI segment	prodrug concn (mM)	hydrolysis (%)
duodenum	1	5
jejunum	1	12
	0.5	20
	0.25	36
ileum	0.5	6
artificial gastric juice	0.5	no hydrolysis
(with pepsin)	0.25	no hydrolysis

[a] The extent of hydrolysis was determined by radioactive measurements of ^{3}H derivatives (represented by the fragments cleaved off from pamidronate, Pro-Phe, and/or Phe) after selective precipitation of calcium-bisphosphonate complex (n = 2, 16 h).

alendronate was rapidly cleared from plasma and either taken up by bone (60−70%) or excreted by the kidney (30−40%). In addition, the half-life of alendronate in bone is very long, more than 200 days in rats. The

Table 5. Inhibition of HAP Formation in 3.87 mM Calcium and 2.32 mM Phosphate Metastable Solution by Pamidronate and Pro-Phe-pamidronate and Inhibition of HAP Dissolution in Vitro (at steady state) by Adsorbed Pamidronate and Pro-Phe-pamidronate

	inhibition of HAP dissolution, % of control		inhibition of HAP formation, % of initial concentration	
compound	0.24 (mM)	0.048 (mM)	1.0 (mM)	0.1 (mM)
NaCl	100.0 ± 8.7	100.0 ± 3.7	18.0 ± 0.5	17.0 ± 2.1
pamdronate	55.7 ± 2.6*	79.4 ± 4.9*	46.9 ± 1.8*	63.8 ± 1.9*
Pro-Phe-Pam	59.1 ± 3.7*	79.5 ± 5.2*	40.5 ± 0.71*	83.8 ± 1.26*

[a] The amount of calcium dissolved in the NaCl group was termed as 100%. *Differences were termed statistically significant by the Kruskal−Wallis and Dunn's tests ($4 \le n \le 10$, mean ± SD, $p < 0.05$).

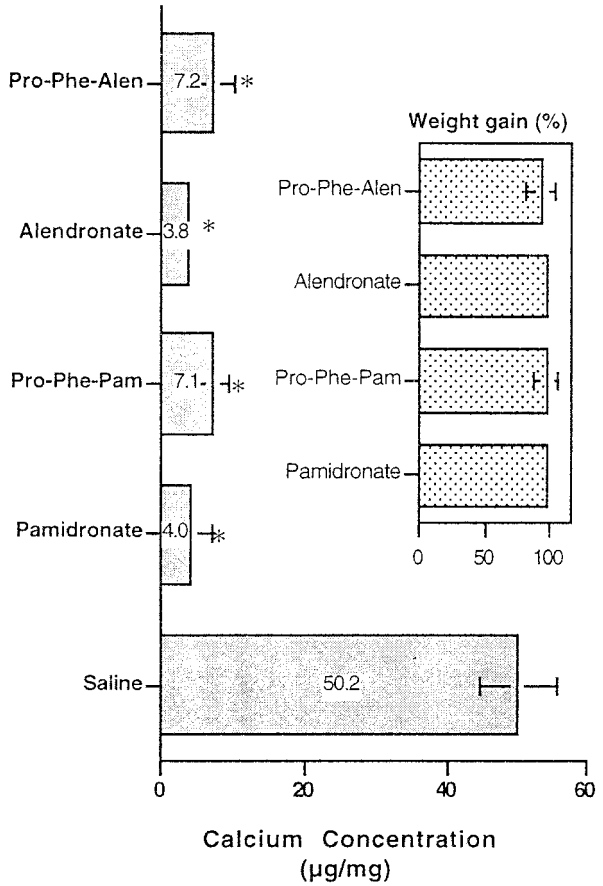

Figure 7. Effect of Pro-Phe-pamidronate and Pro-Phe-alendronate delivered by miniosmotic pumps on bioprosthetic heart valve tissue calcification implanted sc in rats for 14 days. Mini osmotic pumps containing 0.014 M drug solution, delivering test compounds at 1.68 μmol/kg/day, were co-implanted sc with bioprosthetic heart valve tissue (1 × 1 cm). Data are presented as μg calcium/mg dry tissue weight. *Differences were termed statistically significant by the Kruskal−Wallis and Dunn's two-tailed test (mean ± SE, n = 6, $p < 0.05$).

pharmacokinetics of bisphosphonates have been characterized to a limited extent in humans. It is difficult to quantify the concentration of a bisphosphonate in biological fluids, especially in plasma, following therapeutic doses of alendronate due to the rapid clearance and limited analytical methods. Since alendronate and pamidronate are largely deposited in bone where they have a long half-life, their absorption is estimated in animals by measuring the drug concentration in bone tissues.[27] In addition, since renal excretion is the only

route of elimination of the drugs, absorption can also be assessed by measuring the drug concentration in the urine.[27] The results of this study show that the oral administration of the dipeptidyl prodrugs Pro-Phe-pamidronate and Pro-Phe-alendronate resulted in a significant increase of drug absorption. Drug concentration in the urine, following oral administration of the prodrugs Pro-Phe-pamidronate and Pro-Phe-alendronate, was between 2 and 3 times higher than that obtained following administration of the parent drugs (Figure 2). Similarly, the concentration of pamidronate in the tibia from the prodrug was higher than that following oral administration of the parent drug (Figure 2). Alendronate concentration in the tibia following oral administration of Pro-Phe-alendronate revealed the same phenomenon with drug concentration from the prodrugs higher than that following parent drug administration (Figure 2). The bioavailability of Pro-Phe-alendronate was 3.3 (F_{TIBIA}) and 1.9 (F_{URINE}) times higher than that of alendronate. The iv experiment with Pro-Phe-alendronate compared to alendronate revealed 2.5 (bisphosphonate moiety) and 3.4 (peptide moiety) times higher levels in the feces (not quite significant, $p = 0.0931$). The latter finding indicates that there might be excretion of the drug via bile. This finding, coupled with the relatively high levels of the prodrug in soft tissues (kidney and liver) even 24 h post-prodrug administration, as well as the small extent of prodrug reconversion noted after iv administration, suggests that the disposition of the intact prodrug is different from that of the parent drug. Thus the bioavailability calculated according to drug accumulation in bone and urine is not precise, and the actual bioavailability is probably higher.

Although proline and phenylalanine are unpolar (hydrophobic) amino acids, the octanol/buffer partition coefficient of the prodrugs was similar to that of the parent drugs (0.001, $\log P = -3$). Since the prodrug has a similar lipophilicity and 2 times larger molecular weight than the parent drug, the increased absorption of the prodrugs could not be ascribed to increased passive diffusion. The mechanism of the improved absorption of the prodrugs was elucidated by the perfusion and pylorus-ligated rat studies. Previous studies on the absorption of Gly-Gly and Gly-Leu across the gastrointestinal tract in human showed no transport in the stomach but did show the following order of transport activity in the small intestine: jejunum > ileum > duodenum where the active carrier system exists.[28] In situ single-pass perfusion studies showed that the intestinal absorption of Pro-Phe-pamidronate was reduced by Pro-Phe (Figure 5). The competitive inhibition by the dipeptide suggests that Pro-Phe-pamidronate shares a common carrier with the small peptides, hPEPT1. In addition, the extent of absorption of our prodrug from the stomach was significantly less than that observed from the GI tract (Figure 4). Moreover, pamidronate absorption from the stomach was similar to that observed after po administration in control animals (Figure 4). This is further indirect evidence supporting the hypothesis that the increased absorption of the prodrug is due to active transport.

Chong et al.[29] investigated whether there is a quantitative difference between drugs that are absorbed primarily via the dipeptide transporter system and drugs that are absorbed by passive diffusion in the Caco-2 cell line. The conclusion of this study was that the in vitro permeability through Caco-2 cells is not quantitatively predictive of in vivo absorption for peptide-like drugs absorbed via the dipeptide transporter system. Despite this limitation of the Caco-2 system, transport of the prodrugs (dipeptidyl moiety and bisphosphonate moiety) from the ap to bl direction was found to be significantly higher than that of the parent drug. The ap to bl and bl to ap transports of alendronate and pamidronate were similar, which indicates that the transport of the bisphosphonates is passive. The dipeptidyl moiety was transported significantly higher from the ap to bl direction in comparison to the bl to ap direction. The bisphosphonate moiety had an insignificantly better transport from the ap to bl direction. (Table 3). The prodrug's high affinity to the intestinal tissue and the increased transport in Caco-2 cells further support a carrier-mediated transport of the peptidyl prodrugs.

Since it is known that the peptide carrier system has broad specificity and excellent capability, the relatively small increment of prodrug absorption (2−4 times) may suggest that the prodrug inhibits transport. In addition, the absorption was inhibited by an hPEPT1 competitive inhibitor transport supporting the peptidyl carrier mechanism. However, it is plausible to assume that the increased absorption of the prodrugs could be attributed to another active carrier system as well.

The contribution of the hydrolyzed free form should also be considered. Since the levels of ^{14}C (bisphosphonate moiety) were higher than those of ^{3}H (peptide moiety) in the feces (Figure 3), the free form of the drug hydrolyzed in the lumen could not contribute to the prodrug absorption. On the other hand, free drug discharged at the intestinal wall by brush-border enzymes could contribute in the prodrug increased absorption. This is supported by the high affinity of the prodrugs to the intestinal tissue (Figure 6). The free drug at the brush-border will not chelate with calcium or other heavy metals and, therefore, could contribute to the transport.

To be useful as an oral prodrug of bisphosphonates, the peptidyl prodrugs have to withstand enzymatic attacks in the GI tract prior to absorption. The stability of Pro-Phe-pamidronate was studied in artificial gastric juice and in homogenates derived from three regions of the intestine. In the experimental time span, Pro-Phe-pamidronate was stable in artificial gastric juice, suggesting that the prodrug is resistant to pepsin and low pH and might be administered as a plain tablet with no need for entero-coating. As shown in Table 4, Pro-Phe-pamidronate underwent relatively little degradation in the intestinal cell homogenate. The decreased hydrolysis of Pro-Phe-pamidronate in the intestinal mucosal homogenate of the jejunum as the concentration increased may suggest a saturation of the enzyme. The metabolism of the prodrugs in the body fluids/tissues can also be estimated from the analytical results of the different isotopes after oral administration. As expected, the concentrations of the amino acids in the urine and soft tissues, especially in the liver (a metabolic organ), were substantially higher than those of the

bisphosphonates (Figure 3). The high concentrations of the amino acids in the feces (Figure 3) suggest relatively little bioconversion of the prodrugs in the intestinal lumen, thus indicating that the absorption of the prodrugs was achieved with no significant hydrolysis.

The results presented indicate that relatively high concentrations of the peptidyl prodrug may not completely convert to the parent drug after systemic uptake. Therefore examination of the biological activities of the prodrugs takes on added importance. For this purpose two experimental models that are commonly used for predicting and evaluating the anticalcification effects of bisphosphonates were used. In the in vitro model Pro-Phe-pamidronate was found to be as effective as pamidronate in inhibiting HAP formation (Table 5). Good inhibition of HAP formation in vitro implies high activity as a crystal poison in vivo.[30,31] Indeed, the prodrugs significantly inhibited the calcification of tissues implanted subdermally in rats. The mechanism of action of bisphosphonates in inhibiting bioprosthetic heart valve tissue calcification is mainly attributed to a direct physicochemical effect on crystal growth and dissolution ("crystal poison").[32] It can be assumed that the greater the affinity of the bisphosphonate to the crystal, the greater its "crystal poison" effect.

To evaluate the potential antiresorptive properties of the bisphosphonate prodrugs, we examined the effects on inhibition of HAP dissolution at pH 5. In the experimental conditions employed, using a HAP tablet and two concentrations of Pro-Phe-pamidronate, it was possible to detect small differences between the activities of the prodrug in comparison to the parent drug. The prodrug significantly inhibited HAP dissolution (Table 5). To some extent, the in vivo antiresorption effects of the compounds can be predicted on the basis of the results obtained by this model.[33] Classical prodrug is inactive in the parent form; therefore, the prodrug activity results and the low metabolism in vivo suggest that the derivatives may not be behaving as classical prodrugs.

In conclusion, it was shown in this work that the bioavailability of bisphosphonates can be enhanced by using the peptidyl prodrug approach. The increased oral absorption of the prodrugs was mediated by an active carrier-mediated transport.

Experimental Section

3-Amino-1-hydroxypropane 1,1-Bisphosphonic-*1-*^{14}*C* Acid (Pamidronate-*1-*^{14}*C*). 3-Aminopropanoic-*1-*^{14}*C* acid (0.374 g, 4.2 mmol having total activity 1 mCi), water (0.30 mL, 16.4 mmol) and pyridine (1.08 mL, 13.4 mmol) were placed in a flask provided with an efficient condenser and a powerful mechanical stirrer. Dry hydrogen chloride gas was introduced into the mixture with vigorous stirring until saturation. After cooling to 40 °C, phosphorus trichloride (1.25 mL, 14.3 mmol) was added, and the mixture was stirred on a bath gradually heated from room temperature to 95 °C, and then kept at 95 °C for 1 h. Water (0.3 mL) and concentrated hydrochloric acid (0.2 mL) were added, and the mixture was hydrolyzed by heating for 5 h at 135 °C (in the bath). Addition of water (1.5 mL) and 2-propanol (2.0 mL) to the reaction mixture caused crystallization to give 0.765 g (77%) of radioactive pamidronate, pure according to ^{31}P NMR (solvent: water + 10% of pyridine) $\delta = 17.5$ ppm, t, $J = 12.5$ Hz.

4-Amino-1-hydroxybutane 1,1-Bisphosphonic-*1-*^{14}*C* Acid (Alendronate-*1-*^{14}*C*). This synthesis was carried out similarly to the previous experiment using 4-aminobutyric-*1-*^{14}*C* acid (0.39 g, 3.76 mmol having total radioactivity 1 mCi), water (0.30 mL, 16.4 mmol) and pyridine (1.08 mL, 13.4 mmol) to yield 0.682 g (72%) of product, ^{31}P NMR (solvent: $D_2O + Na_2CO_3$, pH = 5) $\delta = 18.0$ ppm, t, $J = 12$ Hz.

L-Prolyl-L-phenylalanylpamidronate (Pro-Phe-pamidronate). *N*,*N*′-Dicyclohexylcarbodiimide (520 mg, 2.52 mmol) was added to a stirred and ice cooled solution of *N-tert*-butoxycarbonyl-L-prolyl-L-phenylalanine (Boc-L-Pro-L-PheOH) (660 mg, 1.82 mmol) and *N*-hydroxysuccinimide (HOSU, 230 mg, 2 mmol) in dichloromethane (CH_2Cl_2, 12 mL). After stirring for 30 min at 0 °C the solution was further stirred for 48 h at room temperature. The precipitated dicyclohexylurea was filtered and washed with CH_2Cl_2 (10 mL). The combined organic solvents were evaporated, and the residue was treated with pentane (25 mL). The crystalline product was filtered and washed with pentane to give 803 mg *N-tert*-butoxycarbonyl-L-prolyl-L-phenylalanine *N*-hydroxysuccinimide ester (Boc-Pro-Phe-OSU). This compound was added to a solution of 3-amino-1-hydroxypropane 1,1-bisphosphonic acid (277 mg, 1.18 mmol) and diisopropylethylamine (0.71 mL, 4.15 mmol) in 1.7 mL 2-propanol and 0.25 mL water. After stirring the solution for 48 h, the solvent was evaporated to afford a syrup which was taken up in water (13 mL) and acetic acid (0.53 mL) resulting in a solution of pH 3. The solution was filtered to remove impurities and evaporated. A 14% solution of benzathine diacetate in water (13 mL), was added to the residue causing the formation of a viscous syrup. After 2 h the supernatant liquid was removed by decantation and a new portion of 13 mL benzathine solution was added. The mixture was then left overnight. The supernatant was decanted again, and the viscous syrup was washed with water (3 × 5 mL) and ether (2 × 12 mL). The washing solutions were removed by decantation. The syrup was dissolved in a solution of water−methanol (1:2, 80 mL) and passed through a column of Dowex 50 (H^+ form, 30 mL). The column was then washed with water−methanol (1:2, 80 mL) until the pH of the eluted liquid was 4−5. The eluted solution was evaporated to give a mixture of *N-tert*-butoxycarbonyl-L-prolyl-L-phenylalanylpamidronate and l-prolyl-L-phenylalanylpamidronate. To this mixture 1.8 mL of trifluoroacetic acid was added to complete the removal of the *tert*-butoxycarbonyl protecting group. After 30 min at room temperature the reaction mixture was evaporated to dryness followed by coevaporation with methanol (5 × 25 mL). The solid obtained was filtered and washed with methanol to yield 388 mg (70%) of L-prolyl-L-phenylalanylpamidronate. NMR (in D_2O with Na_2CO_3, pH = 7): ^{31}P{H} 17.9 ppm; ^{1}H 1.4−2.4 (m, 6H, Pro 2β CH_2, pamidronate β CH_2); 3−3.5 (m, 6H, Pro α CH_2, pamidronate γ CH_2, Phe benzyl CH_2); 4.25 (m, 1H, Pro α CH); 4.52 (m, 1H, Phe α CH); 7.2−7.4 (m, 5H, Phe aromatic). MW calcd 479.36, found by FAB MS 479.8. Anal. ($C_{17}H_{27}N_3O_9P_2 \cdot 2H_2O$) C; H; N. $[\alpha]^{20}_D = -21.9°$ ($c = 2$, in 1% triethylamine in methanol).

L-Prolyl-L-phenylalanylalendronate (Pro-Phe-alendronate). This compound was synthesized using the method described for the synthesis of L-Prolyl-L-phenylalanylpamidronate by adding alendronate (2 g, 8.03 mmol) to a solution of Boc-Pro-Phe-OSU (5.141 g, 11.2 mmol) and diisopropylethylamine (4.2 mL, 28.6 mmol) in 11.4 mL 2-propanol and 1.8 mL water. After stirring the solution for 48 h, the reaction was worked up as previously described to yield 2.09 g (53%) of L-prolyl-L-phenylalanylalendronate. NMR (in D_2O with Na_2CO_3, pH = 7): ^{31}P $\delta = 19.5$ ppm; t, ($J = 12$ Hz); ^{1}H 1.7−1.97 (m, 8H, Pro 2β CH_2, alendronate $\beta + \gamma$ CH_2); 3.02−3.05 (m, 4H, pamidronate γ CH_2, Phe benzyl CH_2); 3.1−3.2 (2H, m, Pro α CH_2) 4.1−4.15 (m, 1H, Pro α CH) 4.2−4.25 (m, 1H, Phe α CH); 7.2−7.4 (m, 5H, Phe aromatic Hs). MW calcd 493.39, found by FAB MS 493.2. Anal. Calcd ($C_{18}H_{29}N_3O_9P_2 \cdot 2H_2O$) C, 40.84; H, 6.28; N, 7.94. Found C, 41.14; H, 6.12; N, 6.6. $[\alpha]^{20}_D = -24.45°$($c = 2$, in 1% triethylamine in methanol).

L-Prolyl-L-phenylalanyl-^{3}H-pamidronate-*1-*^{14}*C* (Pro-Phe-^{3}H-pamidronate-*1-*^{14}*C*). A reaction mixture containing L-phenylalanine-*1-*^{14}*C* (496 mg, 3.0 mmol, total activity 5 mCi) water (1 mL), diisopropylethylamine (0.5 mL), 2-propanol (20 mL), water (2 mL) and *N-tert*-butoxycarbonylprolyl-*N*-hydroxy-

succinimide ester (844 mg, 2.7 mmol) was stirred at ambient temperature for 24 h. The mixture was then filtered and washed with 2-propanol. The filtrate was transferred to a separatory funnel and extracted with dilute phosphoric acid and then with water. After drying, the ethereal solution was evaporated to yield 722 mg (74%) Boc-Pro-PheOH-*^{3}H*. To the dipeptide dissolved in CH_2Cl_2 and cooled in an ice bath, *N*-hydroxysuccinimide (252 mg, 2.19.mmol) and dicyclohexylcarbodiimide (569 mg, mmol 2.76 mmol) were added, and the mixture was allowed to stand at ambient temperature for 48 h. After removing the dicyclohexylurea by filtration, the solution was evaporated, the residue was taken up in pentane, and the precipitate was collected to yield Boc-Pro-Phe-OSU-*^{3}H*. To the activated dipeptide a solution of pamidronate-*1-^{14}C* (480 mg, 2.0 mmol), diisopropylethylamine (1.21. ml), water (0.42 mL) and 2-propanol (2.84 mL) was added, and the solution was stirred at ambient temperature for 48 h. The reaction was worked up as described previously for Pro-Phe-pamidronate to yield 482 mg (50%) of L-prolyl-L-phenylalanylpamidronate, having ^{1}H and ^{31}P NMR spectra identical with those of the "cold" product.

L-Prolyl-L-phenylalanyl-^{3}H-alendronate-*1-^{14}C* (Pro-Phe-^{3}H-alendronate-*1-^{14}C*). To a solution of labeled activated dipeptide Boc-Pro-Phe- OSU-*^{3}H* (918 mg, 2.0 mmol) in CH_2Cl_2, alendronate-*1-^{14}C* (449 mg, 1.8 mmol) in a solution (1.21 mL), consisting of 2-propanol (2.56 mL) and water (0.4 mL) and diisopropylethylamine (1.1 mL, 6.42 mmol) was added. The solution was left to be stirred at ambient temperature for 48 h. The reaction mixture was worked up as described previously to yield 294 mg (30%) L-prolyl-L-phenylalanylalendronate having a ^{31}P{H} NMR spectrum identical with that of the cold product.

iv Administration. The tested compounds and dosages were [^{14}C]pamidronate (1 mg/kg, 1μCi/mg), Pro-[^{3}H]Phe-[^{14}C]-pamidronate (1 mg/kg of pamidronate, ^{14}C 0.5 μCi/mg; ^{3}H 2.6 μCi/mg), [^{14}C]alendronate (1 mg/kg, ^{14}C 1 μCi/mg) and Pro-[^{3}H]Phe-[^{14}C]alendronate (1 mg/kg of alendronate, ^{14}C 0.5 μCi/mg; ^{3}H 2.5 μCi/mg). Male Sabra rats weighing 220−250 g were kept in metabolic cages for 1 week prior to the investigation for acclimatization, with free access to water and food. An indwelling cannula was inserted into the rat's right jugular vein under light ether anesthesia 1 day before drug administration. A dose of 1 mg/kg of the tested compound was dissolved in iso-osmotic vehicle (0.3 mL saline). The rats were euthanized by overexposure to ether 24 h after drug administration, and the tibia, femur, kidney, liver, intestine, spleen, urine and feces were obtained and frozen until assay. Radioactivity levels in tissues were determined by combustion to $^{14}CO_2$ and ^3H_2O by means of a sample oxidizer (Sample Oxidizer, Packard, Meriden, CT) and liquid scintillation counting, using appropriate standard curves (enabling separate determinations of ^{14}C (CO_2) and ^{3}H (H_2O) counts in a mixture). Counting of ^{14}C and ^{3}H enabled differentiation between Pro-Phe-bisphosphonate and/or Phe-bisphosphonate and/or bisphosphonate on one hand, to Pro-Phe and/or Phe on the other.

Oral Administration. The tested compounds and dosages were [^{14}C]pamidronate (10 mg/kg, 1 μCi/mg), Pro-[^{3}H]Phe-[^{14}C]-pamidronate (10 mg/kg of pamidronate, ^{14}C 0.5 μCi/mg; ^{3}H 2.6 μCi/mg), [^{14}C]alendronate (10 mg/kg, ^{14}C 1 μCi/mg) and Pro-[^{3}H]Phe-[^{14}C]alendronate (10 mg/kg of alendronate, ^{14}C 0.5 μCi/mg; ^{3}H 2.5 μCi/mg). Male Sabra rats weighing 250−300 g were kept in metabolic cages for 1 week prior to the investigation for acclimatization, with free access to water and food. Each group of rats (n = 6) received an oral dose of the tested compound by a stomach tube after an overnight fast. 24 h later, the animals were euthanized by an overexposure to ether, and the tibia, femur, kidney, liver, intestine, muscle, urine and feces were removed for radioactivity measurements. Radioactivity levels in tissues were determined by combustion to $^{14}CO_2$ and ^3H_2O by means of a sample oxidizer (Sample Oxidizer, Packard, Meriden, CT) and liquid scintillation counting, using appropriate standard curves (enabling separate determinations of ^{14}C (CO_2) and ^{3}H (H_2O) counts in a mixture). Counting of ^{14}C and ^{3}H enabled differentiation between Pro-Phe-bisphosphonate and/or Phe-bisphosphonate and/or bisphosphonate on one hand, to Pro-Phe and/or Phe on the other.

Bioavailability values of the prodrugs were estimated by the ratios of $^{14}C_{po}/^{14}C_{iv}$ accumulated in bone or urine 24 h following administration corrected for the dose:[8]

$$F_{\mathrm{TIBIA}} = (\text{amount tibia}_{po} \times D_{iv})/(\text{amount tibia}_{iv} \times D_{po}) \times 100$$

$$F_{\mathrm{URINE}} = (\text{amount urine}_{po} \times D_{iv})/(\text{amount urine}_{iv} \times D_{po}) \times 100$$

In a separate experiment the time dependent distribution of Pro-Phe-alendronate and alendronate after oral administration was examined. The distribution of the prodrug and the parent drug in the intestinal tissue was determined after po administration of 10 mg/kg bisphosphonate. Male Sabra rats weighing 250−300 g were kept in metabolic cages for 1 week prior to the investigation for acclimatization, with free access to water and food. Each group of rats (n = 10) received an oral dose of the tested compound by a stomach tube after an overnight fast. The animals (2 rats/time point) were euthanized and the intestinal tissues were excised at 0.25, 0.5, 1.5, 3 and 24 h. Radioactivity levels in intestine (duodenum) tissue were determined by combustion to $^{14}CO_2$ and ^3H_2O by means of a sample oxidizer (Sample Oxidizer, Packard, Meriden, CT) as described above.

Gastric Absorption. The active carrier system is present only in the intestine. To determine site of prodrug absorption, the absorption of pamidronate and Pro-Phe-pamidronate from the stomach was studied in rats following pylorus ligation. The peritoneal cavity was opened by midline incision under ketamine and xylazine anesthesia; the pylorus was ligated; the incision was sutured; and the rats were allowed to recover for a period of 2 h. Pamidronate (10 mg/kg) and Pro-Phe-pamidronate (20 mg/kg, equivalent to 10 mg/kg of pamidronate) were administered orally by stomach tube to 2 rats in each group. Two hours following drug administration the rats were euthanized. The tibia, femur, kidney, liver and spleen were then obtained, and the amounts of the drug and the prodrug were determined as described above.

In Situ Single-Pass Perfusion. The technique was similar to previous reports.[34] The rats (male Sabra rats weighing 200−250 g) were fasted overnight (12−18 h) before each experiment. Anesthesia was induced by im injection of nembutal (0.1 mL/100 g). The rats were kept on a heating slide warmer to maintain their body temperature. The abdominal cavity was opened by a middling incision of 3−4 cm. The jejunum was located and cannulated at 2−4 cm below the ligament of Treitz and about 10 cm distal to the first incision.[34] The tubing between the peristaltic pump and the inlet cannulae was placed in a water bath of 37 °C. After cannulation, the intestinal segment was placed within the abdominal cavity, carefully avoiding crimping or kinking of the segment. The incision was then covered by saline-wetted paper towels. The tested compounds (0.09 mM) were pamidronate (n = 6), alendronate (n = 8), Pro-Phe (n = 4), Pro-Phe-pamidronate (n = 6) and Pro-Phe-alendronate (n = 8). The permeability of Pro-Phe-pamidronate was tested in the absence and in the presence of competitive inhibitors (n = 6, 4 mM Pro-Phe). The jejunal segments were perfused with a Sorensen's phosphate buffer solution (pH 7.4), containing one of the tested compounds, with a flow rate of 0.19 mL/min using a peristaltic pump (Ismatec, Switzerland). Samples were taken for 2 h in 10-min intervals after reaching steady state in the perfused segment (after 30 min) and analyzed by scintillation counting. Due to the double labeling of the compound, neither [^{14}C]- nor [^{3}H]PEG-4000 could be examined to calculate water flux. Therefore, water flux correction was accomplished by measuring the inlet and outlet volume of the perfusate. Water transport below 0.6%/cm of intestinal length was considered to be normal and experiments with higher water transport were not used in the determination of P_{eff}. The concentration ratios used for the permeability calculation were obtained employing the following equation:

$$C_{in}'/C_{out}' = (C_{in}/C_{out}) \times (V_{in}/V_{out})$$

with C_{in}'/C_{out}' as the volume-corrected concentration ratio, C_{out} and C_{in} as the inlet and outlet perfusate concentrations, respectively, and V_{out} and V_{in} as the inlet and outlet volumes of the perfusate, respectively. For all experiments the inlet and outlet volumes were measured with a micropipet (0.5−10 μL) up to a volume of 10 μL. This methodology had previously been evaluated as a feasible substitution for nonabsorptive markers.

The accuracy of the method was verified in preliminary perfusion experiments by comparing water flux calculations based on labeled PEG permeability (without the double-labeled drug) to volume change of the perfusate. Water flux calculation based on [^{14}C]PEG-4000 recovery ($PEG_{rec} = PEG_{out}/PEG_{in} = 98 \pm 5.3\%$) compared to that obtained by using volume change of perfusate was in very good correlation. Moreover, we verified this method of volume correction by determining the effective permeability of various compounds (with different absorption mechanisms and physicochemical properties). The results obtained (L-arginine $P_{eff} = 1.48 \times 10^{-4} \pm 1.581 \times 10^{-5}$, $n = 6$; L-arginine + ouabaine $P_{eff} = 6.73 \times 10^{-5} \pm 4.91 \times 10^{-6}$, $n = 5$; PEG-4000 $P_{eff} = 5.07 \times 10^{-7} \pm 4.13 \times 10^{-7}$, $n = 5$; and colchicine $P_{eff} = 4.82 \times 10^{-5} \pm 6.94 \times 10^{-6}$, $n = 6$) are in accordance with the literature.[35,36] The effective jejunal permeability coefficient (P_{eff}) was calculated according to the following equation:[36]

$$P_{eff} = [Q_{in} \ln(C_{in}/C_{out})]/2\pi rL$$

where Q_{in} is the perfusion flow, C_{in}/C_{out} (dpm/mL) is the volume corrected concentration ratio, and $2\pi rL$ is the transfer surface area, where L is the segment length and r is the radius (0.2 cm). Radioactivity levels in the specimens were determined by liquid scintillation counting, using appropriate standard curves as above.

Transport in the Caco-2 Cell Line. The tested compounds were [^{14}C]mannitol (51.5 mCi/mmol, purchased from New England Nuclear Products, Boston, MA), [^{14}C]alendronate (1.0 μCi/mg), [^{14}C]pamidronate (1.0 μCi/mg), Pro-[^{3}H]Phe-[^{14}C]-alendronate ([^{3}H] 2.3 μCi/mg, [^{14}C] 0.5 μCi/mg), Pro-[^{3}H]Phe-[^{14}C]pamidronate ([^{3}H] 2.6 μCi/mg, [^{14}C] 0.5 μCi/mg) and Pro-[^{3}H]Phe (6.35 μCi/mg).

Cell Culture. Caco-2 cells (obtained from University of California, San Francisco, CA) originating from a human colorectal carcinoma were grown in 75 cm^2 flasks at 7% CO_2. The culture medium consisted of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% NEAA (nonessential amino acids), 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin. For the transport studies cells were seeded at a density of 80 000 cells/ cm^2 onto the rat tail collagen (type I) coated Transwell polycarbonate filters (24-mm diameter, 3.0-μm pore size) (Costar, Cambridge, MA). The culture medium was changed every other day. Transport studies were performed 21−25 days after seeding. Caco-2 cells at passage number of 46−48 were used for experiments.

Transport Studies. Transport studies were carried out in Hank's balanced salt solution (HBSS) (pH 7.4) containing 25 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (Hepes) as previously described.[2] The cells grown on the collagen-coated polycarbonate membranes were rinsed twice with HBSS−Hepes solution before each experiment. At the beginning of the experiment HBSS−Hepes solution was added to the receiver chamber (either apical or basolateral side) and the test solution to the donor chamber (either apical or basolateral side). Bisphosphonates, their dipeptidyl prodrugs and proline-phenylalanine were dissolved in HBSS−Hepes solution to a concentration of 500 μM. The apical and basolateral reservoir volumes were maintained at 1.5 and 2.6 mL, respectively. The monolayers were incubated at 37 °C with stirring. The samples (200 μL) were taken from the receiver chamber at regular intervals up to 4 h and replaced with equal volume of HBSS−Hepes solution.

Epithelial integrity of cell monolayers was routinely assessed by the flux measurements of radiolabeled paracellular marker [^{14}C]mannitol (0.3 μCi/mL). [^{14}C]Mannitol permeability was not more than 4.1×10^{-7} cm/s from the apical to basolateral direction and not more than 6.1×10^{-7} cm/s from the basolateral to apical direction in each experiment. Moreover the TEER values were routinely measured using the epithelial voltammeter (EVOM-G, World Precision Instruments, Sarasota, FL). TEER values were 800 ± 90 Ω·cm^2 the day before permeability studies. The TEER of the cell layers was calculated using the following equation:

$$\text{TEER} = (R_{total} - R_{blanc})\pi r^2$$

where R_{total} is the resistance measured, R_{blanc} is the resistance of control filters without cells, and r is the radius of filter. Radioactivity of the samples was determined using a liquid scintillation counter (WinSpectral 1414 LSC, Wallac Co., Turku, Finland).

Data Analysis. The apparent permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = dQ/dt \cdot 1/(AC_0)$$

where dQ/dt is the permeability rate, A is the surface area of the monolayer, and C_0 is the initial donor solution concentration. For peptidyl prodrugs both the bisphosphonate and proline-phenylalanine parts of the prodrug were quantified in the receiver chamber and separate apparent permeability coefficients were calculated.

Statistical Analysis. The results are expressed as mean ± SD and were statistically evaluated by Mann−Whitney U-test ($p < 0.05$).

Bioconversion. Bioconversion of Pro-[^{3}H]Phe-[^{14}C]pamidronate was studied in intestinal mucosal homogenate and artificial gastric juice. The cytosolic fraction of rat intestinal mucosal was prepared according to the method of Bai et al.[37] with some modification. The small intestine was detached and its length was measured. An ice-cold solution of 0.15 M KCl was pushed gently through the segment to clean out luminal contents. The mucosa was scraped off and weighed, and 5 mL of pH 7.8, 0.15 M Tris buffer was added to 1 g of mucosal tissues. The tissues were homogenized in an ice bath at a speed of 1140 rpm. The homogenate was then centrifuged at 4 °C, 108000*g* for 1 h. The supernatant fraction was harvested as the enzyme source. The reaction solution consisted of 0.5 mL of the supernatant and 0.5 mL of substrate solution (0.25, 0.5 and 1mM prodrug concentration), and the extent of overnight hydrolysis (16 h) at 37 °C was determined. An appropriate amount of 10% (w/v) trichloroacetic acid was used to stop the hydrolysis. A solution of diluted phosphoric acid (pH 2) was added 10 min later to achieve appropriate dilution prior to centrifugation.

The extent of prodrug hydrolysis was determined by selective precipitation of the bisphosphonate as calcium salt. The samples of the intestinal homogenate were combined with 100 μL of $CaCl_2$ solution ($CaCl_2 \cdot H_2O$, 150 mM) to precipitate the bisphosphonate species (pamidronate, Phe-pamidronate or Pro-Phe-pamidronate). In separate experiments we verified that the amino acid Phe and the dipeptide, Pro-Phe, do not precipitate in such conditions, and that the bisphosphonate is completely precipitated. The specimens were centrifuged and the dipeptide/amino acid (Pro-Phe, and/or Phe) amount in the supernatant fraction was determined by radioactivity measurements of ^{3}H derivatives, which represented the fragments cleaved off from pamidronate. The bioconversion of the prodrug was determined from the ratio of ^{3}H counts between the precipitate and the supernatant.

Bioconversion of the prodrug was also studied in simulated gastric juice. The artificial gastric juice solution (GJS) contained (in 1000 mL): 2 g NaCl, 80 mL 1 N HCl, and 3.2 g pepsin at pH of 1.2 ± 0.1. Pro-Phe-pamidronate solution was prepared in distilled water and was added in a 1:1 ratio to the GJS. The prodrug final concentrations were 0.5 and 0.25

mM. After an overnight incubation at 37 °C, the extent of hydrolysis was determined by selective precipitation of the calcium-bisphosphonate complex as described previously.

Inhibition of Hydroxyapatite Precipitation. The inhibition of hydroxyapatite (HAP) precipitation formation in the presence of Pro-Phe-pamidronate (0.1 and 1 mM) and Pro-Phe-alendronate (0.1 and 1 mM) was studied as described previously.[21] In brief, the HAP concentration product of calcium ($CaCl_2 \cdot 2H_2O$) and phosphate (K_2HPO_4) in the incubated solutions was 9 mM^2, calcium 3.87 mM and phosphate 2.32 mM, yielding a molar ratio of $Ca/PO_4 = 1.67$, as in HAP. Each salt solution was prepared in 0.05 M Tris buffer, pH 7.4, and the tested prodrug or drug was dissolved in the phosphate solution. After 24 h of incubation, calcium concentration in the filtrate was determined by atomic absorption spectroscopy.[38]

Inhibition of HAP Dissolution. HAP tablets (300 mg HAP and 0.5% magnesium stearate) were prepared in a carver press (10-mm diameter). The tablets were coated with beewax, leaving only one surface exposed. The tablets were stirred at 37 °C overnight in a solution of Pro-Phe-pamidronate or pamidronate (240 μM and 48 μM in 10 mL Tris buffer, pH 7.4. The HAP tablets with the adsorbed drug were transferred to 10 mL acetate buffer pH 5, and stirred at 37 °C. At each time point, aliquots of 1 mL were analyzed for dissolved calcium by atomic absorption spectroscopy.[38] To maintain constant HAP:solution ratio, the dissolution medium was replenished with 1 mL of acetate buffer after each sampling.

Inhibition of Ectopic Calcification. The anticalcification effect of the prodrugs in-vivo was studied by examining the inhibition of bioprosthetic tissue calcification implanted subdermally in rats.[21,30,39] Bioprosthetic heart valve tissue cusps were prepared from bovine pericardium treated with glutaraldehyde, as described previously.[38] Mini osmotic pumps (Alzet 2001, Alza, Stanford, CA) containing 0.014 M drug solution delivering test compounds at 1.68 μmol/kg/day for 14 days, were placed next to subcutaneous bioprosthetic tissue cusps (1 × 1 cm) implanted in the dorsal part of each ether-anesthetized rat. One tissue cusp, implanted subcutaneously in the abdominal wall of each animal, served as a paired control. An additional group of rats receiving bioprosthetic tissue implants without treatment, served as an unpaired control. Euthanasia was carried out by ether 14 days after implantation, and the amount of calcium in the retrieved tissues was determined by atomic absorption spectroscopy on aliquots of HCl hydrolysates. The amount of calcium was expressed as μg calcium/mg dry tissue weight. The control values represent the average of the data of both control groups since there was no difference between these groups.

Statistics. The various parameters measured were expressed as mean ± SE and were statistically evaluated according to the Mann−Whitney test (one-tailed, $p < 0.05$) or Kruskal−Wallis and Dunn's tests (two-tailed, $p < 0.05$).

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