Brief Articles

Bisphosphonate Prodrugs: Synthesis and in Vitro Evaluation of Novel Clodronic Acid Dianhydrides as Bioreversible Prodrugs of Clodronate

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Received September 15, 1998

P,*P*′-Diacetyl, *P*,*P*′-dibutyroyl, *P*,*P*′-dipivaloyl, and *P*,*P*′-dibenzoyl (dichloromethylene)bisphosphonic acid dianhydride disodium salts (**2a**−**d**) were synthesized and evaluated as novel bioreversible prodrugs of clodronate. The anhydrides were prepared by reacting anhydrous tetrasodium clodronate with a large excess of the corresponding acid anhydride. The dianhydrides **2a**−**d** alone were more lipophilic than the parent clodronate, as determined by drug partitioning between 1-octanol and phosphate buffer at pH 7.4. They also were stable toward chemical hydrolysis in aqueous solutions (pH 7.4 and 2.0). The half-lives for chemical degradation in a buffer solution at 37 °C varied from 0.7 to 286 h and from 15 to 790 h at pH 2.0 and 7.4, respectively. The dianhydrides **2a**,**b**,**d** underwent complete enzymatic hydrolysis to clodronate in 80% serum at 37 °C after 1 min, although **2c** had a half-life of 3.3 h. The aqueous solubility of clodronate decreased considerably in the presence of Ca^{2+} ions. This is most probably due to formation of poorly water-soluble chelates, which may also hinder the oral absorption of clodronate. However, Ca^{2+} ions did not have an effect on the aqueous solubility of clodronic acid dianhydrides, and therefore, these prodrugs may improve oral absorption of the parent drug. In conclusion, these novel dianhydride derivatives may be potentially useful prodrugs of clodronate which, due to their lipophilicity and lack of Ca^{2+} chelating, increase its bioavailability after oral administration.

Introduction

Synthetic methylenebisphosphonates (MBP), which are characterized by a P−C−P bond, are analogues of pyrophosphate,[1] widely used in the treatment of diseases associated with increased bone resorption and bone metastases.[2] Synthetic bisphosphonates are also the most effective inhibitors of osteoclastic bone resorption and, therefore, useful drugs to treat and prevent osteoporosis.[3−6] The exact mechanism for inhibition of bone resorption is still unknown.[6] However, data concerning the means by which osteoclasts can simultaneously remove large amounts of matrix degradation and penetrate into bone have recently been published.[7]

Clodronate is one of the most documented and well-tolerated MBP derivatives.[8] Tetraacidic MBPs, such as clodronate, are highly hydrophilic due to their ionization at physiological pH. This results in poor membrane permeability and oral bioavailability (i.e., 1−2%[9,10]), which is further decreased in the presence of calcium ions.[2,8] Masking one or more ionizable groups of clodronate by using the prodrug approach would increase the lipophilicity of the molecule and could also decrease its complexation with divalent metal cations.

To our knowledge, prodrugs of clodronate have not been reported, which is most probably due to their difficult chemistry and the problematic chemical properties of clodronate. Various tetraalkyl[11,12] and partial esters[13] of clodronic acid had been studied in order to modify the physicochemical properties of clodronate. However, these esters did not release the parent clodronate by chemical or biochemical conversion, and thus, they do not fulfill the criteria of prodrug.[14] In the present study, we have synthesized novel clodronic acid dianhydrides and evaluated their in vitro properties as potential bioreversible prodrugs of clodronate.

Results and Discussion

Chemistry. Four clodronic acid dianhydride derivatives with different steric factors (acetyl, butyroyl, pivaloyl, and benzoyl promoieties) were synthesized. An attempt to synthesize *P*,*P*′-dihexanoyl (dichloromethylene)bisphosphonic acid dianhydride did not succeed with that method. The clodronic acid dianhydrides **2a**−**d** (Scheme 1) were prepared by reacting anhydrous tetrasodium clodronate with a large excess of the corresponding acid anhydride at temperatures ranging between 100 and 135 °C. The reaction was fully completed in 24 h for **2a**,**d**. Compound **2b** was achieved after 3 × 120 h heating and **2c** after 2 × 120 h heating with a selectivity of 80−90% (see Experimental Section for details). The dianhydrides **2a**−**d** were purified with ether, water, or a water−alcohol mixture to yield 74%, 53%, 38%, and 63% of theoretical, respectively.

Aqueous Solubility. The dianhydrides **2a**−**d** possess lower aqueous solubility than clodronate (397 mg/mL) at pH 7.4, and the solubility decreased in the order: **2a**

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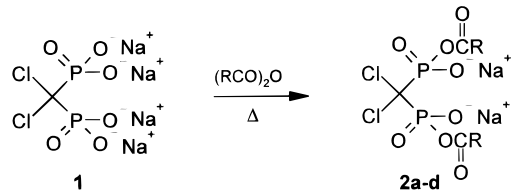
10.1021/jm9810809 CCC: $18.00
Published on Web 04/06/1999

Scheme 1



1 **2a-d**

2a ; R = CH_3
2b ; R = $(CH_2)_2CH_3$
2c ; R = $C(CH_3)_3$
2d ; R = C_6H_5

> **2b** ≫ **2c** > **2d** (Table 1). The absorption of clodronate is hindered by both its formation of poorly water-soluble Ca^{2+} complexes[15] and its low lipophilicity. A crystal structure and ab initio studies have shown earlier bidentate metal bonding in clodronate−calcium−water complexes.[16] The effect of Ca^{2+} ions on the aqueous solubility of clodronic acid dianhydride **2d** and clodronate was determined at pH 6.0. The concentration of clodronate decreased from 894 μg/mL (no calcium added) to 123 μg/mL when 100 mM Ca^{2+} was added to the solution (Figure 1). The concentration of **2d** in buffer solution was not affected by Ca^{2+} ions (Figure 1), which verifies that masking two ionizable groups of clodronate significantly decreases the complexation of clodronate with cations. This may result in an improved oral absorption of clodronate.

Apparent Partition Coefficient. The log P_{app} values were determined using the 1-octanol−pH 7.4 buffer system. The log P_{app} value for clodronate was not successfully determined due to its very hydrophilic character. However, it can be estimated to be less than −5.4, which has been reported for clodronic acid monoethyl ester at pH 7.4.[17] Clodronic acid dianhydrides **2a**−**d** are significantly more lipophilic than the parent clodronate at pH 2.0 and 7.4 (Table 1). The change of promoiety group in the structure did not have a significant effect on log P_{app} values, and thus, more than two phosphonic acid groups of clodronic acid may need to be substituted in order to increase further the log P_{app} values.

Hydrolysis in Aqueous Solution. The hydrolysis of **2a**−**d** to clodronate followed first-order kinetics (Figure 2). The dianhydrides **2a**−**d** were more stable toward chemical hydrolysis at pH 7.4 than at pH 2.0 (Table 1). The dianhydrides **2c**,**d** were significantly more stable in aqueous solution than **2a**,**b** (Table 1). This is most probably due to the resonance stabilization of benzoyl promoieties in **2d** and the steric hindrance of pivaloyl promoieties in **2c**.

Hydrolysis in Human Serum. Rates of enzymatic hydrolysis of **2a**−**d** were determined in 80% human serum (pH 7.4) at 37 °C. These hydrolyses followed first-order kinetics (Figure 2) and were substantially faster than the chemical hydrolyses (Table 1). The dianhydrides **2a**,**b**,**d** hydrolyzed rapidly (<1 min) and completely to clodronate. Compound **2c** was more resistant toward enzymatic hydrolysis, having a half-life of 3.3 h, which is most probably due to its more hindered structure.

Conclusion

The present clodronic acid dianhydrides, which are shown to be novel bioreversible prodrugs of clodronate, are more lipophilic than the parent clodronate, stable against chemical hydrolysis, and hydrolyzed enzymatically to clodronate in human serum. To our knowledge, the clodronic acid dianhydrides are the first reported bioreversible prodrugs of clodronate with the potential to improve the oral bioavailability of clodronate.

Experimental Section

Chemistry. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker AM 400 spectrometer operating at 400.1, 100.6, and 162.0 MHz, respectively; TSP was used as an internal standard for ^{1}H and ^{13}C measurements and 85% H_3PO_4 used as an external standard for ^{31}P measurements. Normal $^3J_{HH}$ couplings are indicated by the letter "*J*", and all *J* values are given in Hz. All solvents and reagents were high-purity reagent-grade materials, and the acetonitrile was distilled before use. Synthesis and characterization of tetrasodium clodronate has been reported earlier.[18] HPLC measurements were performed with a Merck LaChrom HPLC system consisting of model L-7250 programmable autosampler, model L-7100 HPLC pump, model D-7000 interface module, and model D-7000 HPLC system manager (Hitachi Ltd., Tokyo, Japan). A Sedex 55 evaporative light-scattering detector (Sedere, Vitry-Sur-Seine, France) was used. The analytical column used was a Kromasil 100 RP-C8 (250 × 4.6 i.d., 5 μm; Higgins Analytical Inc., Mountain View, CA). The eluent consisted of 3% methanol and 97% 0.10 M ammonium acetate buffer (pH 4.6) containing 0.23 M *n*-butylamine. After 1.5 min of elution, the organic concentration was increased linearly from 3% to 60% for **2d** and from 3% to 70% for **2b**,**c** in 5 min. pK_a values were measured with Mettler Toledo DL 50 (Schwerzenbach, Switzerland).

***P,P′*-Diacetyl (Dichloromethylene)bisphosphonate Disodium Salt (2a).** Anhydrous tetrasodium clodronate (**1**) (15.0 g, 45.1 mmol) and acetic anhydride (150.0 mL, 1589.8 mmol) were heated in an oil bath at 135 °C for 24 h. The mixture was chilled to 5 °C and allowed to stand overnight in the cold. The mixture was filtered, and the solids were washed several times with small portions of ether (130 mL) and dried to give **2a** as a white solid (12.5 g, 74%). pK_{a1} = 2.4, pK_{a2} = 6.4. NMR (D_2O): δ_H 2.21 (6H, s); δ_P 2.91 s; δ_C 172.48 t (virtual triplet,[19] due to poor signal/noise relation two small signals are covered), 77.37 t ($^1J_{CP}$ = 146.8), 24.85 q+t (virtual triplet). Anal. Calcd ($C_5H_6Cl_2Na_2O_8P_2$): C, 16.10; H, 1.62. Found: C, 15.99; H, 1.90.

***P,P′*-Dibutyroyl (Dichloromethylene)bisphosphonate Disodium Salt (2b).** Anhydrous **1** (1.0 g, 3.0 mmol) and butyric anhydride (10.0 mL, 61.1 mmol) were heated in an oil bath at 110 °C for 120 h and isolated as **2a**. The preceding reaction procedure was repeated a total of three times to give **2b** as a white solid (0.68 g, 53%). NMR (D_2O): δ_H 2.49 (4H, t, *J* = 7.3), 1.65 (4H, m), 0.95 (6H, t, *J* = 7.49); δ_P 3.15 s; δ_C 175.08 t (virtual triplet), 77.64 t ($^1J_{CP}$ = 146.6), 39.95 t+t (virtual triplet), 20.45 t, 15.62 q. Anal. Calcd ($C_9H_{14}Cl_2Na_2O_8P_2$): C, 25.20; H, 3.29. Found: C, 23.26; H, 3.02.

***P,P′*-Dipivaloyl (Dichloromethylene)bisphosphonate Disodium Salt (2c).** Anhydrous **1** (1.33 g, 4.0 mmol), trimethylacetic anhydride (15.0 mL, 73.9 mmol), and acetonitrile (30 mL) were heated in an oil bath at 100 °C for 120 h and isolated as **2a**. The preceding reaction procedure was repeated a total of two times to give the crude product, which was further washed with 35% 2-propanol (15 mL) and dried to give **2c** as a white solid (0.70 g, 38%). pK_{a1} = 2.3, pK_{a2} = 5.6. NMR (D_2O): δ_H 1.25 (18H, s); δ_P 3.75 s; δ_C 179.95 t (virtual triplet), 77.84 t ($^1J_{CP}$ = 147.1), 42.78 t (virtual triplet), 28.88 q. Anal. Calcd ($C_{11}H_{18}Cl_2Na_2O_8P_2$): C, 28.90; H, 3.97. Found: C, 26.21; H, 3.92.

***P,P′*-Dibenzoyl (Dichloromethylene)bisphosphonate Disodium Salt (2d).** Anhydrous **1** (1.0 g, 3.0 mmol), benzoic anhydride (8.0 g, 35.4 mmol), and acetonitrile (20.0 mL) were refluxed for 24 h and isolated as **2a**. The product was mixed with cold water (10.0 mL) for 5 min and filtered. The preceding water wash (5.0 mL) was repeated, and the product was further washed with acetonitrile (10 mL). The solids were dried

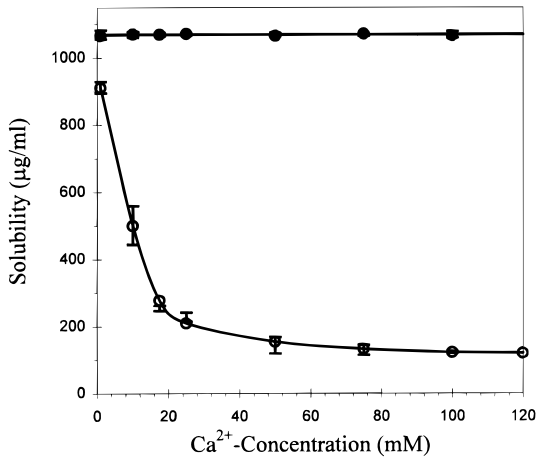


Figure 1. Effect of concentration of Ca^{2+} ions on aqueous solubility of clodronate (○) and **2d** (●) at pH 6.0 at room temperature (mean ± SD, $n = 3$).

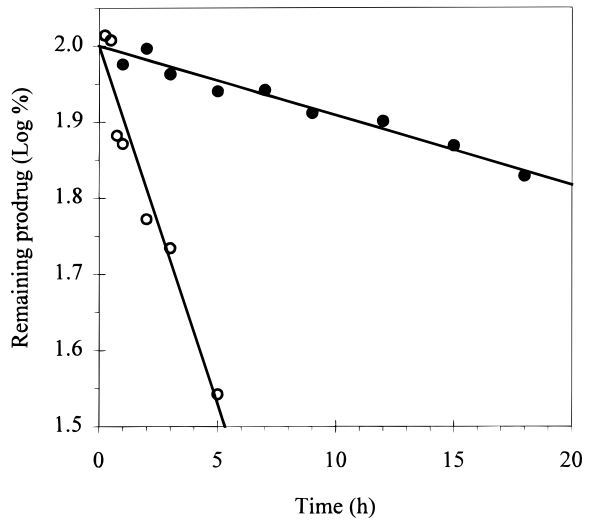


Figure 2. Pseudo-first-order plots for the hydrolysis of **2c** in phosphate buffer (0.05 M, pH 7.4) (●) and in 80% human serum (pH 7.4) (○) at 37 °C.

to give **2d** as a white solid (0.99 g, 63%). $pK_{a1} = 2.2$, $pK_{a2} = 6.4$. NMR (D_2O): δ_H 8.10 (4H, d, $J = 8.0$), 7.65 (2H, t, $J = 7.3$), 7.45 (4H, t, $J = 7.8$); δ_P 3.85 s; δ_C 166.87 t (virtual triplet), 137.32 d, 133.51 d, 131.54 (2C), 80.78 t ($^1J_{CP} = 140.7$). Anal. Calcd ($C_{15}H_{10}Cl_2Na_2O_8P_2$): C, 36.25; H, 2.03. Found: C, 36.01; H, 2.45.

pK_a Values. The pK_a values of **2a**−**d** were determined in water at room temperature using potentiometric titration. An appropriate amount of each prodrug (20−50 mg) was dissolved in water and titrated to pH 2.0 with 0.1 N HCl following back-titration to pH 10 with 0.1 N NaOH.

Aqueous Solubility. The aqueous solubility of **2a**−**d** was determined in phosphate buffer (50 mM, $\mu = 0.15$, pH 7.4) at room temperature. An excess amount of each compound were added to phosphate buffer, and the suspensions were vortexed for 30 min. pH of suspensions was determined during the solubilization and adjusted, if necessary. After equilibration, suspensions were centrifuged and filtered through a 0.45-μL membrane and analyzed by HPLC.

Effect of Ca^{2+} Ions on Aqueous Solubility. A 5 mM solution of clodronate tetrahydrate was prepared in sodium acetate buffer (50 mM, pH 6.0). Calcium chloride ($CaCl_2 \cdot 2H_2O$) was dissolved in the same acetate buffer to achieve the following calcium concentrations: 2, 20, 35, 50, 100, 150, and 200 mM; 750 μL of the 5 mM clodronate solution was added to 750 μL of each calcium-containing solution and mixed for 30 min. After centrifugation, the clodronate concentrations were determined by HPLC using the method described earlier.[14] The concentrations obtained were compared to the concentrations of a sample containing 750 μL of 5 mM clodronate solution and 750 μL of sodium acetate buffer (no calcium chloride added), which was handled in the same manner. All determinations were carried out in triplicate. A 4.4 mM solution of **2d** was prepared in sodium acetate buffer (50 mM, pH 6.0). A 5 mM solution was not prepared due to the limited aqueous solubility of **2d**. The solubility determination was carried out by the same procedure described for clodronate. The HPLC determination of **2d** was carried out by an isocratic HPLC method using a UV detector set to 240 nm. The mobile phase was a mixture of 50 mM ammonium acetate buffer of pH 5.8 (92%), containing 60 mM butylamine and 80% acetonitrile (8%). The flow rate was 1 mL/min, and the analytical column was a Purospher RP-18 (125 × 4 i.d., 5 μm; Merck, Darmstadt, Germany).

Apparent Partition Coefficients. The apparent partition coefficients (log P_{app}) for **2a**−**d** were determined in a 1-octanol and phosphate buffer (50 mM, $\mu = 0.15$) system at pH 2.0 and 7.4. An appropriate amount of each prodrug (0.8−1.5 mg) was dissolved in 1.0 mL of buffer solution and shaken vigorously with 5.0 mL of 1-octanol for 1 h (**2a**−**d**; pH 7.4) or for 5 min (**2a**,**b**; pH 2.0). After shaking, the phases were separated by centrifugation. The concentrations of **2a**−**d** in the aqueous phase, before and after shaking, were determined with HPLC. All determinations were carried out in triplicate.

Hydrolysis in Human Serum. An appropriate amount of each prodrug (6.0−25.0 mg) was dissolved in one volume of phosphate buffer (50 mM, μ 0.15, pH 7.4) at 37 °C, and four volumes of preheated human serum were added. Samples were withdrawn at suitable intervals and deproteinized with an equal volume of methanol, and after mixing and centrifugation, the appropriate amount of the supernatant was evaporated to dryness under a stream of nitrogen and redissolved in water. The concentrations of dianhydride and clodronate were determined by HPLC. The pseudo-first-order rate constants for the hydrolysis in serum were calculated from the slopes of linear plots of the logarithm of remaining prodrug against time.

Hydrolysis in Aqueous Solution. The hydrolysis of **2a**−**d** was studied in phosphate buffer (50 mM, μ 0.15, pH 2.0 and 7.4) at 37 °C. Each prodrug (12 mg) was dissolved in preheated phosphate buffer, and at suitable intervals, samples were withdrawn and analyzed by HPLC. The pseudo-first-order rate constants for the hydrolysis in buffer were calculated from the

Table 1. Apparent Partition Coefficients (log P_{App}; mean values, $n = 3$), Aqueous Solubility (S) at Room Temperature (pH 7.4), and Rate Data ($t_{1/2}$, k_{obs}) of Clodronic Acid Dianhydrides in Phosphate Buffer Solution (pH 2.0 and 7.4) and in 80% Human Serum (pH 7.4) at 37 °C

				rate data (phosphate buffer)					
	log P_{app}		S (mg/mL)	pH 2.0		pH 7.4		rate data (80% serum)	
compd	pH 2.0	pH 7.4	pH 7.4	$t_{1/2}$	k_{obs} (min^{-1})	$t_{1/2}$	k_{obs} (min^{-1})	$t_{1/2}$	k_{obs} (min^{-1})
2a	−1.4	−2.2	190.4	45 min	1.6×10^{-2}	15.2 h	7.6×10^{-4}	*a*	*a*
2b	−1.9	−2.3	171.6	49 min	1.4×10^{-2}	31.3 h	3.7×10^{-4}	*a*	*a*
2c	−2.0	−2.3	23.5	8.6 h	1.3×10^{-3}	32.9 days	1.5×10^{-5}	3.3 h	3.5×10^{-3}
2d	−1.5	−2.3	6.0	11.9 days	4.0×10^{-5}	9.8 days	4.9×10^{-5}	*a*	*a*

[a] Completely hydrolyzed during 1 min.

slopes of linear plots of the logarithm of remaining prodrug against time.

Acknowledgment. This work was financially supported by the Academy of Finland and the Technology Development Centre (Finland). The elemental analyses were performed at the University of Joensuu (Finland). We would like to thank Mrs. Helly Rissanen and Maritta Salminkoski for their skillful technical assistance.

Supporting Information Available: ^{1}H and ^{31}P NMR spectra for **2a**−**d**. This information is available free of charge via the Internet at http://pubs.acs.org.

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JM9810809