Supporting Information

Thermodynamics of Bisphosphonate Binding to Human Bone: A Two-Site Model

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Materials & Methods:

Isothermal titration calorimetry

ITC experiments were performed on a VP-ITC calorimeter (Microcal, Inc., Northampton, MA). Human bone tissue (non-demineralized bone powder, 45-250 μm particle size,) was obtained from the Pacific Coast Tissue Bank (2400 S. Flower St., 5th Floor, Los Angeles, CA, USA. Phone number: 800-745-0034 Fax number: 213-745-3031). Typically, the bisphosphonate samples were made by dissolving 10 mM bisphosphonate in 100 mM PH=7.0 HEPES buffer. The pH of the solution was then readjusted to 7.0. Bone samples were prepared by suspending 50 mg bone in 2 mL 100 mM HEPES buffer at pH=7.0. We calculated the final concentration of bone sample in the calorimeter sample cell as approximately 40.4 mM, based on the observation that bone contains 60% hydroxyapatite (monomer FWt=502 g/mol) [1]. Approximately 48 mg bone sample was transferred into the 1.42 mL sample cell, therefore, the final concentration is:

$$\frac{60\% * 48 \text{ mg}}{502 \text{ mg/mmol} * 1.42 \text{ mL}} = 40.4 \text{ mM}$$

All samples and buffers were degassed prior to calorimetry. The ITC experiments were conducted by sequentially titrating 10 μL bisphosphonate sample from a 250 μL syringe (rotating at 460 or 490 rpm) into the 1.42 mL sample cell containing bone at 37 °C. The initial delay was set to 300 s, each injection was 24 s long and was separated by a 300 s delay between injections so that the heat of reaction reached baseline prior to the next injection. Longer delays between injections did not affect the results obtained, consistent with full binding of the bisphosphonates into all accessible sites. For all experiments, the

heats of dilution were obtained just prior to the bone experiments, and were obtained by titrating the bisphosphonate sample into 100 mM HEPES buffer (pH=7.0) using the same ITC parameters. The heat of dilution was then used as a reference, being subtracted from the experiment with bone by using Origin software (from Microcal, Inc., Northampton, MA). The subtraction curves were analyzed by using non-linear regression methods, and could be well fitted using either a one-site binding model (**4**,**6**,**8**,**10**-**12**) or a two-site binding model (**1-3**, **5**, **7**, and **9**) [2].

Computational approach to ΔG, ΔH, and ΔS prediction:

We treat ΔG, ΔH, and ΔS for bisphosphonate binding to bone each as a linear combination of variables:

$$\vec{y} = A\vec{x} \quad \text{where} \quad y_i = \sum^{N} a_{i,n} x_n$$

For example, the total free energy ($\vec{y}$) of binding is a linear combination of the ΔG values for: NH_3^+, $PO_3(1)$, $PO_3(2)$ together with OH, and a general hydrophobic side-chain (1-H or phenyl group) term:

$$\Delta G_{Tot} = \sum \Delta G_{individual} = \Delta G_{NH_3^+} + \Delta G_{PO_3(1)} + \Delta G_{PO_3(2)+OH} + \Delta G_{hydrophobic}$$

The second PO_3 and the OH group contribution are not separable (since all compounds that bind to site B have both $PO_3(2)$ and the 1-OH group), so we grouped their contributions together as $\Delta G_{PO_3(2)+OH}$.

Applying a pseudo-binary descriptor linear regression analysis, we searched for the best fit, or linear combination of functional group individual free energies, that can predict the total free energy. The vector and matrix rank data is arranged in the order Site A, then Site B.

$$y = \Delta G_{Tot} = \begin{bmatrix} -6.5 \\ -5.7 \\ -5.4 \\ -5.2 \\ -4.7 \\ -4.9 \\ -5.0 \\ -4.8 \\ -5.6 \\ -5.1 \\ -4.7 \\ -5.1 \\ -10.4 \\ -9.3 \\ -8.4 \\ -7.3 \\ -8.0 \\ -7.7 \end{bmatrix} = \begin{bmatrix} \Delta G\ Site\ A\ compound\ 1 \\ \Delta G\ Site\ A\ compound\ 2 \\ \Delta G\ Site\ A\ compound\ 3 \\ \Delta G\ Site\ A\ compound\ 4 \\ \Delta G\ Site\ A\ compound\ 5 \\ \Delta G\ Site\ A\ compound\ 6 \\ \Delta G\ Site\ A\ compound\ 7 \\ \Delta G\ Site\ A\ compound\ 8 \\ \Delta G\ Site\ A\ compound\ 9 \\ \Delta G\ Site\ A\ compound\ 10 \\ \Delta G\ Site\ A\ compound\ 11 \\ \Delta G\ Site\ A\ compound\ 12 \\ \Delta G\ Site\ B\ compound\ 1 \\ \Delta G\ Site\ B\ compound\ 2 \\ \Delta G\ Site\ B\ compound\ 3 \\ \Delta G\ Site\ B\ compound\ 5 \\ \Delta G\ Site\ B\ compound\ 7 \\ \Delta G\ Site\ B\ compound\ 9 \end{bmatrix}$$

The columns of A are ordered:

$$A = \begin{bmatrix} \Delta G_{NH_3^+} & \Delta G_{1,PO_3} & \Delta G_{2,PO_3+OH} & \Delta G_{hydrophobic} \\ \vdots & \vdots & \vdots & \vdots \\ \vdots & \vdots & \vdots & \vdots \\ \vdots & \vdots & \vdots & \vdots \end{bmatrix}$$

We set A as a pseudo-binary matrix of values, mirroring the relative contribution of each functional group to the total free energy:

$$A = \begin{vmatrix} 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 0.33 & 1 & 0 & 0 \\ 0.33 & 1 & 0 & 1 \\ 0.03 & 1 & 0 & 0 \\ 0.03 & 1 & 0 & 1 \\ 0.03 & 1 & 0 & 0 \\ 0 & 1 & 0 & 1 \\ 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 1 \\ 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 1 \\ 1 & 1 & 1 & 0 \\ 1 & 1 & 1 & 0 \\ 0.33 & 1 & 1 & 0 \\ 0.03 & 1 & 1 & 0 \\ 0.03 & 1 & 1 & 0 \\ 0 & 1 & 1 & 0 \end{vmatrix}$$

in which 1 = an interaction is present, 0 = it is absent.

To take account of the different pK_a values of the ammonium (**1**,**2**), imidazolium (**3**,**4**), and pyridinium (**5**,**6**) side-chains, we took the ammonium to be fully protonated (1), the pyridiniums to have pK_a of 6.7 so at pH=7 there is a 0.33 mole fraction of these species present, and the pK_a of the pyridiniums to be 5.5, resulting in a 0.03 fraction protonated species (at pH=7.0), implying that Coulombic interactions of these charged side-chains dominate the bone binding interaction.

Using a multiple linear regression analysis, we determined:

$$\vec{x} = \begin{vmatrix} -1.60 \\ -4.82 \\ -3.06 \\ -0.0538 \end{vmatrix}$$

That is, the relative ΔG contributions for each functional group are:

$$\begin{aligned} NH_3^+ &= -1.60 \\ PO_3(1) &= -4.82 \\ PO_3(2)+OH &= -3.06 \\ \text{hydrophobic} &= -0.0538 \end{aligned}$$

Statistics for this regression when applied to all 18 (12 Site A, 6 Site B) compounds are:

$R^2 = 0.95$

F-value = 89 with $p < 0.00001$

Error variance estimate = 0.19

ΔH and –TΔS calculations:

Using the same pseudo-binary matrix and multiple linear regression methods as used to calculate ΔG, we determined the thermodynamic group properties for ΔH and -TΔS to be:

ΔH:

$$\begin{aligned} NH_3^+ &= -0.0925 \\ PO_3(1) &= -1.33 \\ PO_3(2)+OH &= 2.01 \\ \text{hydrophobic} &= 0.934 \end{aligned}$$

-TΔS:

$$\begin{aligned} NH_3^+ &= -1.51 \\ PO_3(1) &= -3.49 \\ PO_3(2)+OH &= -5.07 \\ \text{hydrophobic} &= -0.989 \end{aligned}$$

The corresponding statistics for the two (ΔH, -TΔS) linear regressions were:

ΔH:

$R^2 = 0.57$

F-value = 6.1 with $p < 0.0070$

Error variance estimate = 0.71

-TΔS:

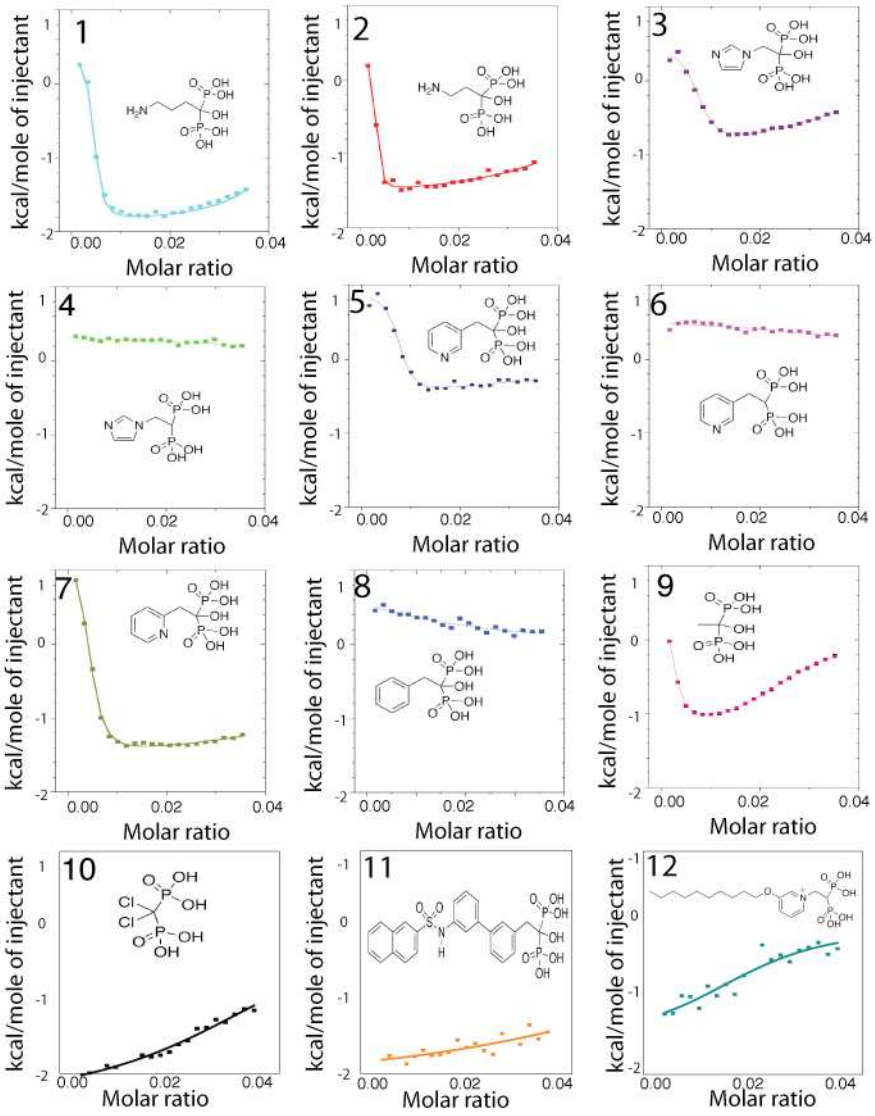
$R^2 = 0.91$

F-value = 47 with $p < 0.00001$

Error variance estimate = 0.73

The ΔH statistics are worse than the ΔG, -TΔS statistics since the ΔH range is smaller. Eliminating the hydrophobic term had overall no effect on the ΔG statistics (R^2=0.95), since ΔH (=0.93 kcal mol^{-1}) ≈ TΔS (0.99 kcal mol^{-1}).

Figure S1: Isothermal calorimetry results for **1-12** binding to human bone



Molar ratio values calculated assuming that bone is 60% hydroxyapatite (monomer FWt = 502 g/mol).

Table S1: Isothermal titration calorimetry results for **1-12** binding to human bone showing ΔG, ΔH, ΔS, N (the site occupancy), together with (where applicable) their standard deviations (from two or three independent experiments).

	Site A				Site B			
	ΔG (kcal/mol)	**ΔH (kcal/mol)**	**ΔS (cal/K/mol)**	**N1**	**ΔG (kcal/mol)**	**ΔH (kcal/mol)**	**ΔS (cal/K/mol)**	**N2**
1 (alendronate)	-6.5(±0.13)	-1.7(±0.16)	15(±0.94)	0.040(±0.0035)	-10.4(±0.70)	0.60(±0.18)	35(±2.9)	0.0038(±0.00010)
2 (pamidronate)	-5.7(±0.072)	-1.4(±0.17)	14±(0.78)	0.040(±0.0085)	-9.3(±0.30)	0.41(±0.097)	31(±0.67)	0.0022(±0.00034)
3 (zoledronate)	-5.4(±0.20)	-1.0(±0.0035)	14(±0.67)	0.034(±0.0032)	-8.4(±0.51)	0.54(±0.086)	29(±1.9)	0.0063(±0.0006)
4 (deoxyzoledronate)	-5.2(±0.095)	0.49(±0.15)	18(0.79)	0.048(±0.0054)	a	a	a	a
5 (risedronate)	-4.7(±1.0)	-0.69(±0.44)	13(±4.3)	0.028(±0.0053)	-7.3(±0.89)	0.89(±0.19)	27(±2.3)	0.0080(±0.0012)
6 (deoxyrisedronate)	-4.9	0.56	18	0.052	a	a	a	a
7 (ortho-risedronate)	-5.0(±0.48)	-1.3(±0.57)	12(±3.3)	0.057(±0.036)	-8.0(±0.23)	0.68(±0.63)	28(±1.42)	0.0048(±0.0017)
8 (desazarisedronate)	-4.8(±0.48)	0.86(±0.36)	18(±0.38)	0.02(±0.0033)	a	a	a	a
9 (etidronate)	-5.6(±0.17)	-1.4(±0.13)	14(0.95)	0.019(±0.0044)	-7.7(±0.37)	0.74(±0.088)	27(±0.90)	0.0023(±0.00023)
10 (clodronate)	-5.1	-2.4	8.7	0.040	a	a	a	a
11 (BPH-675)	-4.7	-2.1	8.4	0.078	a	a	a	a
12 (BPH-715)	-5.1	-1.6	11	0.020	a	a	a	a

[a] Second binding site not present

Table S2: Experimental and computed ΔG, ΔH, and -TΔS values for **1**-**12** binding to Sites A, B.

Compounds	**Site**	**ΔG (expt, kcal)**	**ΔG (calc, kcal)**	**ΔH (expt, kcal)**	**ΔH (calc, kcal)**	**-TΔS (expt, kcal)**	**-TΔS (calc, kcal)**
1	A	-6.4558	-6.4199	-1.7175	-1.4221	-4.7384	-4.9978
2	A	-5.6842	-6.4199	-1.4000	-1.4221	-4.2842	-4.9978
3	A	-5.4241	-5.3493	-1.0035	-1.3601	-4.4206	-3.9894
4	A	-5.1566	-5.4032	0.49010	-0.42620	-5.6466	-4.9782
5	A	-4.6985	-4.8700	-0.69330	-1.3323	-4.0052	-3.5379
6	A	-4.8800	-4.9238	0.56000	-0.39850	-5.4467	-4.5266
7	A	-5.0015	-4.8700	-1.2517	-1.3323	-3.7499	-3.5379
8	A	-4.7671	-4.8759	0.86400	-0.39570	-5.6311	-4.4815
9	A	-5.6040	-4.8221	-1.3730	-1.3296	-4.2315	-3.4927
10	A	-5.0560	-4.8759	-2.3650	-0.39570	-2.6908	-4.4815
11	A	-4.7050	-4.8221	-2.0890	-1.3296	-2.6164	-3.4927
12	A	-5.0950	-4.8759	-1.5610	-0.39570	-3.5340	-4.4815
1	B	-10.406	-9.4841	0.59550	0.58570	-11.002	-10.070
2	B	-9.3487	-9.4841	0.40700	0.58570	-9.7557	-10.070
3	B	-8.3762	-8.4135	0.54300	0.64760	-8.9187	-9.0611
5	B	-7.3342	-7.9342	0.88600	0.67540	-8.2202	-8.6096
7	B	-8.0108	-7.9342	0.67540	0.67540	-8.6862	-8.6096
9	B	-7.6600	-7.8863	0.74100	0.67820	-8.4010	-8.5644

Full reference for references 7 and 11 in the manuscript:

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