Bisphosphonates (BPs) are the drug of choice for treating bone diseases such as osteoporosis, Paget's disease, and metastatic bone disease. BPs with nitrogen-containing side chains (N-BPs) are known to act as inhibitors for farnesyl pyrophosphate synthase (FPPS), a key enzyme in the mevalonate pathway. In this study, we evaluated the effect of different side chains on the binding affinity of BPs to human FPPS using calorimetric techniques. Differential scanning calorimetry (DSC) was used to determine the thermal unfolding of FPPS in the presence of BPs. The addition of a series of clinically available BPs increased the structural stability of human FPPS by preferential binding, as indicated by an increase in the FPPS unfolding temperature. The magnitude of the increase was correlated with in vivo antiresorptive efficacy, suggesting that the stabilization of FPPS underlies the inhibitory effect of the BPs. Isothermal titration calorimetry (ITC) experiments were performed to evaluate the binding thermodynamics of BPs against human FPPS. Analysis of the binding energetics revealed that over 30 years of optimization practiced by different pharmaceutical companies has enhanced the enthalpic contribution as well as binding affinity of BPs. The larger enthalpic contribution observed for newer, more potent BPs derives from both improved hydrogen bonding interactions and shape complementarity based on comparisons of our results with available structure information.

Key words isothermal titration calorimetry; thermodynamics; drug discovery; enthalpy; differential scanning calorimetry

Osteoporosis is a bone disease which leads to increased bone fragility and fracture risk. In osteoporosis, bone mineral density is reduced, bone microarchitecture deteriorates, and both the amount and variety of proteins in the bone are altered. The most common cause of this disease is increased bone turnover with excessive bone resorption that exceeds bone formation. Bisphosphonates (BPs) are a class of drugs widely used to treat diseases characterized by bone resorption, such as osteoporosis, Paget's disease, and metastatic bone disease.\(^1\)\(^-\)\(^4\) The two phosphate groups in the P–C–P structure of BPs are known to be important for BP interaction with a molecular target in the osteoclast and for the high affinity to bone mineral, both of which are required for BPs to inhibit bone resorption.\(^4\)\(^-\)\(^6\) As shown in Fig. 1, side chains attached to the central carbon of the P–C–P allow for a wide range of possible chemical structures of clinically available drugs.\(^3\) However, while a wide variety of side chain structures has evolved from 30 years of research and development at different pharmaceutical companies, what underlies progression towards quality compounds in terms of thermodynamics of binding to a target remains unclear.

First-generation BPs such as etidronate and clodronate have non-nitrogenous (non-N) side chains and act as pyrophosphate analogues. These non-N-BPs are metabolized to non-hydronezygable cytotoxic ATP analogues that accumulate intracellularly in osteoclasts, resulting in the induction of osteoclast apoptosis.\(^7\) In contrast, second- and third-generation BPs are N-BPs, having an alkyl-amino side chain and a nitrogenous heterocyclic side chain, respectively. While known to have high antiresorptive potency, these compounds are not metabolized in the osteoclast cytosol. Instead, they inhibit the enzyme farnesyl pyrophosphate synthase (FPPS) by mimicking one of its substrates, dimethylallyl pyrophosphate (DMAAPP).\(^5\)\(^-\)\(^15\)

FPPS (EC 2.5.1.10) is a homodimeric protein and a key regulatory enzyme in the mevalonate pathway.\(^9\) The enzyme catalyzes sequential condensation of isopentenyl pyrophosphate (IPP) and DMAAPP to generate geranyl pyrophosphate (GPP), which is then condensed with an additional IPP to farnesyl pyrophosphate (FPP). Inhibition of FPPS by N-BPs leads to a reduction in the levels of FPP and its product, geranylgeranyl pyrophosphate (GGPP). These products are required for the prenylation of small guanosine 5'-triphosphatases (GTPases) such as Ras, Rho, and Rab family proteins, which are necessary for osteoclast function.\(^9\)

A number of studies have clarified the interactions of N-BPs and their analogues with target FPPS.\(^5,12\)\(^-\)\(^15\) Crystallographic analyses of \textit{Escherichia coli} FPPS in complexes with IPP/non-cleavable DMAAPP analogue and with IPP/risedronate have confirmed an inhibition model in which N-BPs inhibit FPPS by occupying the DMAAPP binding site.\(^5\) Subsequent to this finding, a rather large volume of structural information of N-BP/FPPS complexes has been accumulated.\(^16\)\(^-\)\(^22\) Calorimetric techniques such as differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) have also been employed to rank the potency of a wide range of BPs.\(^17,18,21\)\(^-\)\(^23\) DSC can provide information such as the effect of drug binding on the structural stability of target protein,\(^24\)\(^-\)\(^26\) while ITC is the only technique that can directly measure the binding energetics of a biological process (protein–ligand, protein–protein, protein–DNA, etc.).\(^27,28\) The binding thermodynamics of a series of BPs against FPPS from \textit{Trypanosoma cruzi} (parasite that causes Chagas disease) and \textit{Trypanosoma brucei} (parasite that causes sleeping sickness) have been investigated using ITC.\(^17,18,25\) In these studies, the formation of the complex between BPs and FPPS was discussed in detail by comparing binding energetics with X-ray crystal structures.\(^17,29\) The forces driving the binding of N-BPs to FPPS have been shown to differ from those driving the binding to human FPPS.\(^17\)
However, thermodynamics data on BPs binding to human FPPS are available for only a few BPs. Here, we examined the interaction of a wide range of clinically important BPs with human FPPS using DSC and ITC. As an important feature, ITC allows us to precisely determine the Gibbs free energy, enthalpy, entropy, and heat capacity changes associated with binding. Such thermodynamic profiles of the binding are helpful in drug discovery. Recent studies have also suggested the importance of enthalpy-driven optimization when exploring drug candidates. For example, Freire et al. have examined the complete thermodynamics information of two drug classes: statins and human immunodeficiency virus-1 (HIV-1) protease inhibitors. Their findings suggested that later best-in-class compounds are enthalpically better optimized than original first-in-class compounds. Further, it has been reported for HIV-1 protease inhibitors that compounds with higher binding enthalpies can achieve not only higher binding affinities but also better selectivity and drug resistance profiles. However, studies of this type, using a comprehensive set of clinically applicable drugs, remain scarce. In the present study, we aimed to determine the binding thermodynamics of a set of representative BPs and address the forces driving the binding by dissecting their thermodynamics into enthalpy and entropy contributions. We expect this retrospective work to provide a good example on how optimization of drug-target interactions has progressed during drug discovery practice in terms of binding thermodynamics.

**Experimental**

**Bisphosphonates** Ibandronate (Lot. No. 23916613, purity 99.9%) was purchased from LKT Laboratories, Inc. (Saint Paul, Minnesota, U.S.A.). Etidronate, clodronate, pamidronate, alendronate, risedronate, zoledronate, and minodronate were synthesized in-house. MS, 1H-NMR, and elemental analysis were used to confirm the compound structures. The purity of those compounds was determined by elemental analysis to an accuracy of within ±0.3%.

**Construction of the Expression Plasmid** The expression plasmid for human FPPS fusion protein was constructed as follows: The fragment encoding the human FPPS (74–419) domain was amplified using the oligonucleotides hFPP-f (5'-CCT CTA GAC ATA TGG ATG TTT ATG CCC AAG-3') and hFPPS-r (5'-AGA CTC GAG ATT ACT TTC TCC GCT TGT AGA-3'), digested using NdeI and XhoI, and finally cloned into pGBHPS using the same sites.

**Protein Expression and Purification** Human FPPS was expressed in E. coli at 25°C as a GB1-fusion protein. The GB1 and hexahistidine tags and a PreScission protease cleavage site were fused to the N-terminus of FPPS. The protein was grown with E. coli. in 2xYT medium. Protein expression was induced by the addition of isopropyl-1-thio-β-galactopyranoside (IPTG) to a final concentration of 1 mM. The cells were cultured overnight at 25°C. The GB1- and hexahistidine-tag fused human FPPS was purified using a Ni-NTA resin (Qiagen N. V., Venlo, Limburg, Netherlands) and the GB1 and hexahistidine tags were removed by PreScission protease (GE Healthcare U.K. Ltd., Little Chalfont, Buckinghamshire, England). The sample was further purified using a Superdex 200 gel filtration column (GE Healthcare). The total yield of human FPPS was 13.5 mg/900 mL.

**Differential Scanning Calorimetry** Heat capacities following the denaturation of FPPS in the absence or in the presence of IPP and/or BPs were measured as a function of temperature using a high-precision Microcal VP-capiillary DSC (GE Healthcare). Thermal denaturation scans were conducted from 20 to 100°C at a scan rate of 1°C/min. All experiments were performed with freshly prepared FPPS (10 µM) in...
a buffer of 25 mM Tris–HCl, pH 7.5, 25 mM NaCl, and 2 mM MgCl₂. Final concentrations of IPP and BPs added to FPPS were 500 µM. Data were analyzed using Origin 7.0 software (OriginLab, Northampton, MA, U.S.A.) that was provided with the instrument.

Isothermal Titration Calorimetry The enthalpy changes (ΔH) and the affinities (K_a) upon binding of a series of BPs against the IPP/FPPS complex were measured by using a high-precision Microcal Auto-iTC200 system (GE Healthcare). Obtained values were used for calculating the Gibbs free energy (ΔG) and the entropy (ΔS) changes according to the equations K_a=e^(-ΔG/RT) and ΔG=ΔH−TΔS.

The binding thermodynamics of a series of BPs were determined by injecting approximately 200 µM of BPs into a calorimetric cell containing approximately 20 µM of FPPS prebound to 500 µM of IPP. All solutions contained within the calorimetric cell and injector syringe were prepared in the same buffer, PBS, pH 7.4, with 2 mM MgCl₂. Each experiment was performed by one injection of 0.8 µL followed by 27 injections of 1.4 µL with a 500-s interval between each injection. The heat evolved after each injection was obtained from the integration of the calorimetric signal, and the data were analyzed using a single-binding-site model implemented in the ORIGIN software package described above.

Results and Discussion

Thermal Stability of FPPS DSC was employed to evaluate the effect of different BP side chains on the thermal stability of human FPPS. In this study, we used the unfolding midtransition temperature (T_m) to rank the binding ability of BPs against FPPS.24–26 We assumed that the stabilization of FPPS is consistent with the preferential binding of BPs to the native FPPS structure. The results are shown in Fig. 2 and summarized in Table 1. In the absence of IPP, FPPS exhibited a thermal unfolding transition at a temperature of 52.7 °C, and the addition of IPP increased the thermal stability of native FPPS, as indicated by the higher denaturation temperature of 67.5 °C.

Blockade of FPPS enzymatic activity by N-BPs has been reported to result in intracellular accumulation of the IPP substrate.12 Therefore, binding of BPs to human FPPS most likely represents in vivo inhibition when IPP is present in the system. As such, we compared the bindings against human FPPS of a series of BPs in the presence of excess IPP. As shown in Fig. 2, the addition of first-generation BPs (clodronate and etidronate) only slightly affected the FPPS stability, which is consistent with the fact that these non-N-BPs are not actually FPPS inhibitors.7 In contrast, large T_m increases were observed when second- and third-generation BPs (N-BPs) were mixed with the IPP/FPPS complex. In particular, the addition of third-generation BPs such as risedronate, zoledronate, and minodronate30 was found to induce T_m increases of almost 20 °C. These results clearly indicate favorable binding affinities of these third-generation N-BPs against FPPS. The ranking of BPs based on descending unfolding temperature is as follows: minodronate>zoledronate>risedronate>ibandronate>alendronate>pamidronate>etidronate>clodronate. This trend is in good agreement with DSC data previously reported for N-BPs.22

Binding Thermodynamics of BPs The effects of different side chains of BPs on the binding to human FPPS in the presence of IPP were examined using high-sensitivity ITC. The binding of some N-BPs to the IPP/FPPS complex has been reported to be an extremely slow event due to the ‘slow, tight binding’ character.13,21 We observed this same phenomenon and therefore used longer spacing times between injections (500 s) than usual to precisely integrate each signal. Typical traces of calorimetric titrations of the IPP/FPPS complex with BPs are shown in Fig. 3, and all results are summarized in Table 2. Interestingly, even though FPPS is not their actual target molecule, etidronate and clodronate nevertheless bind to the IPP/FPPS complex, albeit with relatively low binding affinities of 1.3 × 10⁷ M⁻¹ (K_d=7.9 µM) and 1.93 × 10⁷ M⁻¹ (K_d=5.2 µM), respectively. The corresponding Gibbs free energies of the bindings were −7.0 kcal/mol for etidronate and −7.2 kcal/mol for clodronate. Decomposing the binding thermodynamics revealed that these two BPs have unfavorable binding enthalpies (+1.7 kcal/mol for etidronate and +1.3 kcal/mol for clodronate), indicating that the binding of first-generation BPs is entropically driven. This result clearly reflects the non-specificity of these first-generation BPs to FPPS. By replacing the non-nitrogenous side chain with alkyl-amino groups, second-generation BPs (pamidronate, alendronate, and ibandronate) improved the binding enthalpies.
by almost 2 kcal/mol, resulting in a 15-fold gain in binding affinity.

Further improvement in binding enthalpies was observed for third-generation BPs, which have nitrogenous heterocyclic side chains. Risedronate, for example, binds to the IPP/FPPS complex with a binding enthalpy of $-7.2$ kcal/mol, which is a much more favorable value than those observed for the 2nd-generation BPs. This gain in binding enthalpy contributed to the increased binding affinity of risedronate of $5.3 \times 10^6$ M$^{-1}$ ($K_d=189$ nM). The binding enthalpy of zoledronate was slightly less favorable ($\Delta H=-6.9$ kcal/mol) compared with risedronate. However, improved binding entropy ($-T\Delta S=-2.6$ kcal/mol) fully compensated for this enthalpic loss, resulting in slightly better binding affinity for zoledronate ($K_d=8.51 \times 10^5$ M$^{-1}$ or $K_d=118$ nM). ITC experiments showed that minodronate had the best binding enthalpy ($\Delta H=-9.0$ kcal/mol) and highest binding affinity in the series ($K_d=1.34 \times 10^7$ M$^{-1}$ or $K_d=75$ nM).

Figure 4 shows the denaturation temperatures of the IPP/FPPS/BP ternary complex obtained from DSC plotted against the binding Gibbs free energies obtained from ITC. A good correlation was observed between the two ($R^2=0.98$). Ranking the N-BPs by these calorimetric data showed good agreement with enzyme inhibition data using recombinant human FPPS and with antiresorptive potency in vivo.$^{13}$ Although the denaturation temperature can successfully rank the potency of a drug in this case, the relationship between the binding affinity and magnitude of the observed temperature shift is not always a simple correlation.$^{26}$

The thermodynamic signatures of the BPs considered in this study are listed in order of launch year$^{39}$ (Fig. 5), and we can note a clear pattern in progression of drug optimization. First-generation non-N-BPs, launched in the late 1970s to mid 1980s, have weak potency, scoring a $K_d$ value in the micro molar range. Second-generation non-N-BPs saw $K_d$ values rise to 200–600 nm. Finally, third-generation N-BPs launched in the late 1990s to the present have $K_d$ values in a much lower nanomolar range than previous generations. This improved potency correlates with improved binding enthalpies.
Remarkably, minodronate, the most potent BP in the series, has a strong binding enthalpy (−9.0 kcal/mol) that accounts for most of its binding affinity. As noted above, Freire et al. previously examined the complete thermodynamic information of two drug classes: statins and HIV-1 protease inhibitors. In those studies, enthalpy appeared as the dominant driving force in the second-generation or best-in-class drugs. Our results demonstrate the same trend for BPs: newer, more potent drugs are enthalpically more optimized. Altogether, as has been suggested previously, our results argue the importance of monitoring thermodynamics balance of the drug/target interaction to efficiently produce best-in-class compounds.

**Origin of Enthalpy Gain**  A more favorable binding enthalpy can be obtained by better van der Waals interactions and/or by better hydrogen bonding. Better van der Waals interactions can be achieved by improving the shape of the drug molecule to better fit the binding cavity. A well-placed hydrogen bond can make a favorable enthalpic contribution on the order of −4 to −5 kcal/mol, a property that will be directly reflected in the binding affinity if enthalpy/entropy compensation is not present. Thus, an in-depth understanding of drug/target interactions can be achieved when the binding thermodynamics are considered in connection with structure-based information.

Analysis of the N-BP-human FPPS complex structure has already been reported. Using this information, we considered the origin of the enthalpy gain observed in newer N-BPs (Fig. 5). An enlarged view of the N-BP side chains packed into the binding cavity of FPPS is shown in Fig. 6. The IDs for the PDB files used in this figure are 2F89 (pamidronate), 2F92 (alendronate), 2F94 (ibandronate), 1YV5 (risedronate), 2F8C (zoledronate), and 3B7L (minodronate). Analyses of the crystallographic structures of the six complexes indicate no significant differences in the mode of binding, conformation or interaction of the P–C–P moiety when they interact with FPPS. In all complexes, the P–C–P moiety faces the conserved aspartate rich motifs and establishes the same interaction with FPPS via a cluster of three Mg$^{2+}$ ions. It is evident, then, that the difference in binding thermodynamics (enthalpy/entropy balance) should be attributed to differences in the side chains.

The side chain nitrogen of pamidronate and alendronate can only make single hydrogen bonds with FPPS, which is reflected by the relatively small enthalpic contribution to their

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**Fig. 4. Correlation between Denaturation Temperature of the IPP/FPPS/BP Complex Obtained by DSC and Binding Gibbs Free Energies of BPs against the IPP/FPPS Complex Obtained by ITC**

A linear relationship was found ($R^2=0.98$).

**Fig. 5. Enthalpic and Entropic Contributions to the Binding Affinity of BPs**

Corresponding binding thermodynamics are listed in the order of the launched year according to the database, thomson reuters integrity. Minodronate is launched only in Japan. Blue, green, and red bars represent the Gibbs energy of binding ($\Delta G$), the enthalpy change ($\Delta H$), and the entropy change ($-T\Delta S$) at 25°C, respectively.
in terms of binding thermodynamics. This combination of methods will significantly reduce the time required to reach a quality enthalpy-driven compound.

Conclusion

In this study, we examined the interaction of a wide range of clinically available BPs against human FPPS using calorimetric techniques and provided thermodynamics information for this important class of drugs. The results presented here provide another comprehensive data set (in addition to data on statins and HIV-1 protease inhibitors) demonstrating how thermodynamics balance—particularly the enthalpic contribution—has been optimized during drug development. In all cases, progression towards the best-in-class compound is associated with gradual improvement in the enthalpic contribution. These retrospective analyses emphasize the importance of monitoring binding thermodynamics (enthalpy–entropy balance) during lead discovery and optimization to efficiently identify best-in-class compounds.

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References


Fig. 6. Surface Representations of the Side Chain of N-BPs Packed into the Binding Cavity of FPPS


