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A General Strategy for Targeting Drugs to Bone

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Abstract: *Targeting drugs to their desired site of action can increase their safety and efficacy. Bisphosphonates are prototypical examples of drugs targeted to bone. However, bisphosphonate bone affinity is often considered too strong and cannot be significantly modulated without losing activity on the enzymatic target, farnesyl pyrophosphate synthase (FPPS). Furthermore, bisphosphonate bone affinity comes at the expense of very low and variable oral bioavailability. FPPS inhibitors were developed with a monophosphonate as a bone-affinity tag that confers moderate affinity to bone, which can furthermore be tuned to the desired level, and the relationship between structure and bone affinity was evaluated by using an NMR-based bone-binding assay. The concept of targeting drugs to bone with moderate affinity, while retaining oral bioavailability, has broad application to a variety of other bone-targeted drugs.*

Nitrogen-containing bisphosphonates (N-BPs) such as zoledronate (**1**, ZOL) and pamidronate (**3**; Scheme 1) are widely used drugs for treating bone loss associated with diseases such as osteoporosis, Paget's disease, and bone metastases.[1] Their remarkable safety and efficacy stem in part from their high affinity to bone mineral, which enables drug accumulation at the desired site of action, where they specifically inhibit osteoclasts by blocking farnesyl pyrophosphate synthase (FPPS).[1] FPPS catalyzes the formation of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) in the mevalonate pathway (see Scheme S1 in the Supporting Information). Recent crystallographic analyses of human FPPS have shown that N-BPs are in fact DMAPP mimetics, with the bisphosphonate moiety very effectively replacing the pyrophosphate group of the substrate as an anchor to the three active-site magnesium ions.[2,3]

Bisphosphonate FPPS inhibitors are generally very safe and effective drugs that display extremely rapid and strong binding to bone mineral, with association rates in the order of minutes and dissociation rates in the order of years.[4] Nevertheless, FPPS inhibitors with no or variable affinity to bone could be therapeutically interesting: For the treatment of non-bone diseases, such as breast cancer,[5a,b] multiple myeloma,[5c] progeria[5d] and parasitic diseases,[5e] more lipophilic FPPS inhibitors devoid of any bone affinity would be useful. On the other hand, for the discovery of improved drugs to treat bone diseases, FPPS inhibitors with weaker and tuneable bone affinity could provide an interesting therapeutic option. Such FPPS inhibitors might have several advantages over bisphosphonates: they could have higher oral bioavailability, they could distribute more evenly into the skeleton, and they could reduce adverse effects such as osteonecrosis of the jaw.[6]

For bisphosphonates, the pharmacophores for FPPS inhibition and bone binding are identical, so bisphosphonates with significantly reduced bone affinity have not been identified in spite of tremendous efforts.[7,8] Our recently discovered allosteric FPPS inhibitors, which bind to a different site on the enzyme and are devoid of any bone affinity,[9] are attractive tools for the treatment of FPPS-related non-bone diseases. For the treatment of bone diseases with compounds with improved properties, we report herein the attachment of a monophosphonate functionality as a bone-affinity tag to allosteric inhibitors of FPPS. Taking advantage of our NMR-based bone binding assay,[10] we show that, in sharp contrast to N-BPs, the bone affinity of these compounds can be tuned to the desired degree, independently from their inhibitory potency towards FPPS. Targeting a drug to the diseased tissue or organ is an attractive concept for improving efficacy and reducing adverse effects. The work described herein provides a practical approach to conferring a suitable degree of bone affinity on a drug candidate, and it may therefore become a general strategy to improve the safety and efficacy of drugs acting on bone.[10,11]

Our research was initially sparked by the serendipitous discovery that AMP397 (**4**, Becampanel; Scheme 1), a drug previously in phase II clinical trials for the treatment of epilepsy, displayed moderate affinity to bone mineral, as shown by quantitative whole-body autoradioluminography and confirmed by our NMR-based hydroxyapatite (HAP) binding assay (see Figure 1 and Figure S1 in the Supporting Information). AMP397 is a monophosphonate but it has a significant oral bioavailability of 22% in mice and approximately 50% in humans.[12] Furthermore, AMP397 has shown preclinical and clinical efficacy as an anti-epileptic drug, thus indicating that it can cross biological membranes and even the blood–brain barrier. In addition, some other monophosphonates, such as the antibiotic fosfomycin, are clinically used drugs with oral bioavailability. Taken together,

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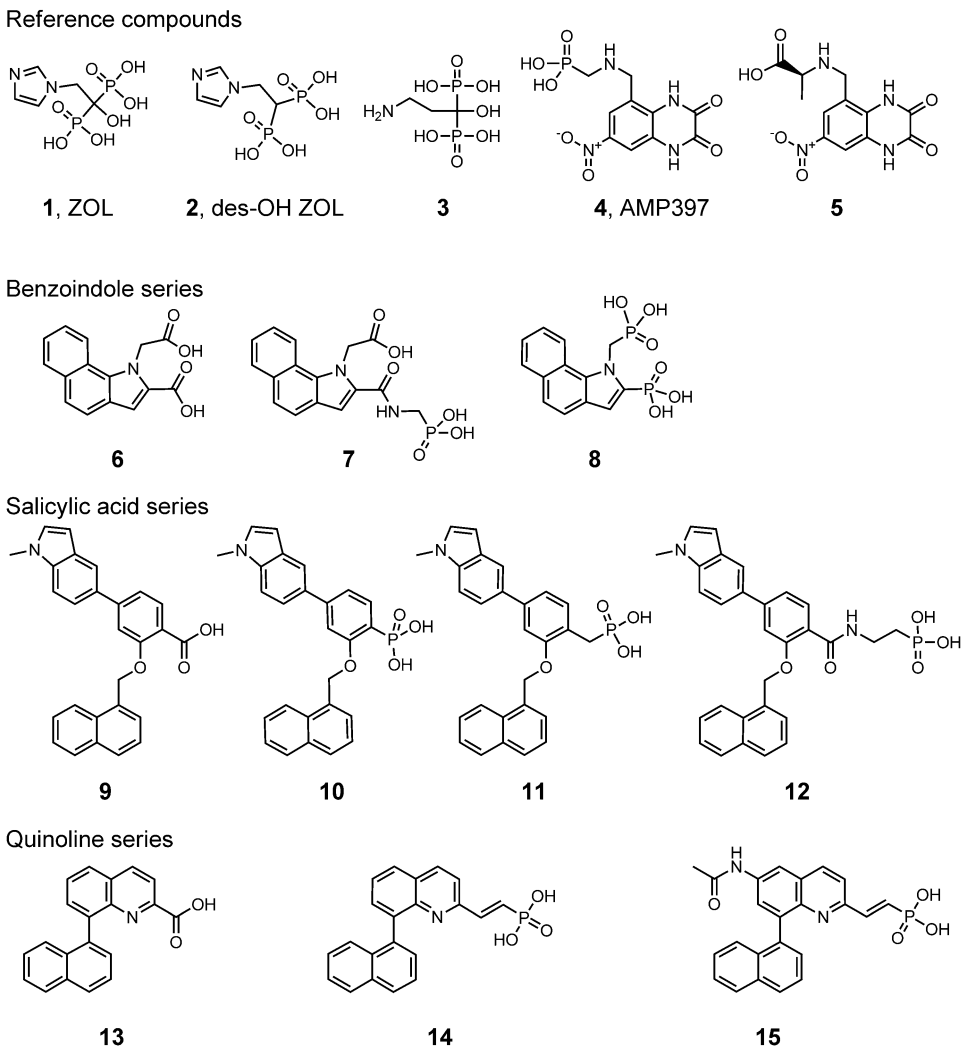

Scheme 1. Structures of the compounds investigated in this study.

these observations suggested that bone affinity can be reconciled with oral bioavailability and significant plasma membrane permeability, and moreover, that a monophosphonate functionality could be a suitable bone-affinity tag.

In a first step, we investigated the bone-binding propensity of AMP397 (**4**) and its carboxylic acid analogue **5**, in comparison to the reference bisphosphonate ZOL (**1**) and its dehydroxy analogue **2**. To this end, our NMR-based assay provided a robust and rapid means to detect and quantify affinity to bone powder or hydroxyapatite (HAP), the main mineral component of bone.[10] In this study, we used HAP, since very similar results were obtained with bone powder and HAP, but the latter showed higher homogeneity and reproducibility.[10] The NMR-based bone binding assay can be run as a direct binding assay or in a competition format, from which relative affinities to bone can be determined, as described.[10,13] Figure 1 A shows the direct-binding format for AMP397, which demonstrates its binding to HAP (see the Supporting Information for experimental details). By contrast, compound **5**, an AMP397 analogue with a carboxylate instead of the phosphonate group, did not show any evidence of HAP binding, thus clearly demonstrating that the monophosphonate functionality is responsible for HAP affinity (Figure 1 B). In the presence of ZOL and limiting amounts of HAP (competition format), AMP397 no longer showed any HAP binding (Figure 1 C), thus indicating at least 10-fold weaker binding of AMP397 compared to ZOL. However, compound **2**, a ZOL analogue with reduced bone affinity, only showed 8-fold stronger binding to HAP as compared to AMP397 (Figure 1 D). Since the binding affinity of **2** to HAP is 2.3-fold weaker than that of ZOL,[10] we concluded that the HAP binding affinity of AMP397 is about 18-fold weaker than that of ZOL or other closely related 1-hydroxy bisphosphonates. Notably, and in spite of this decrease in bone affinity, AMP397 accumulation in bone can still be detected by quantitative whole-body autoradioluminography 72 h after AMP397 administration (Figure S1 in the Supporting Information).

In a second step, we devised a strategy for grafting a monophosphonate functionality onto an allosteric inhibitor of FPPS, such as the benzoindole derivative **6**. On the basis of the available X-ray structures,[9] we identified three options: 1) direct replacement of a carboxylic acid substituent by a phosphonate group, 2) the same but with a spacer, or 3) attachment of a phosphonate group at a suitable solvent-exposed position. Only the first two options were pursued in this project, because the latter would entail an unnecessary increase in molecular weight as well as in the total negative charge of the molecule. Figure 2 illustrates these three options in the context of the crystal structure of the FPPS complex with **6**.

We then synthesized **7**, the readily accessible 2-phosphonomethylcarbamoyl derivative of **6**, and assessed its FPPS inhibitory activity and HAP binding affinity. Gratifyingly, **7** was only slightly weaker ($IC_{50} = 0.4$ μM) as an FPPS inhibitor than its parent compound **6** ($IC_{50} = 0.2$ μM), and it showed significant HAP binding (Figure S2 C). This promising result prompted us to investigate the generality of this strategy by testing it with two recently identified, distinct series of

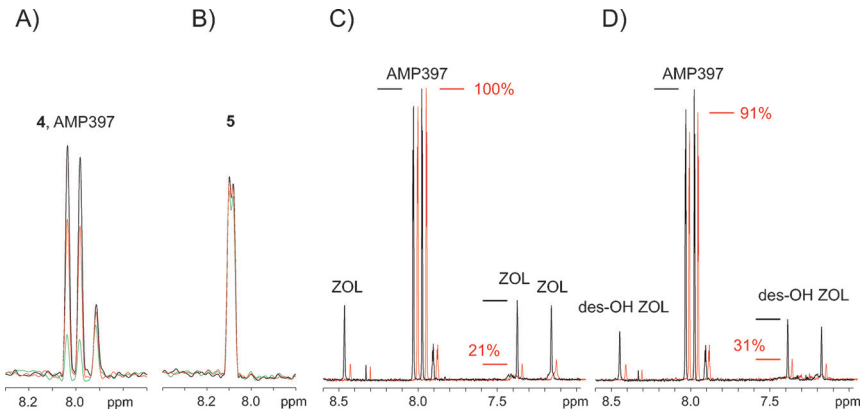

Figure 1. Quantification and characterization of the bone binding affinity of AMP397 (**4**) by the NMR-based HAP binding assay.[10] A) The direct binding assay with increasing amounts of HAP (red and green spectra) demonstrates AMP397 binding to HAP. B) Replacement of the AMP397 monophosphonate group with a carboxylate functionality abolishes HAP binding. C) The competition assay shows no detectable AMP397 binding to HAP in the presence of ZOL. Hence, AMP397 binding to HAP is at least 10-fold weaker than that of ZOL. D) The competition assay with **2**, the dehydroxy analogue of ZOL with a 2.3-fold reduced affinity to HAP,[10] shows 9% AMP397 bound to HAP and 69% **2** bound to HAP. The affinity to HAP of AMP397 is therefore about 8-fold weaker than that of dehydroxy-ZOL **2**, or 18-fold weaker than that of ZOL **1**.

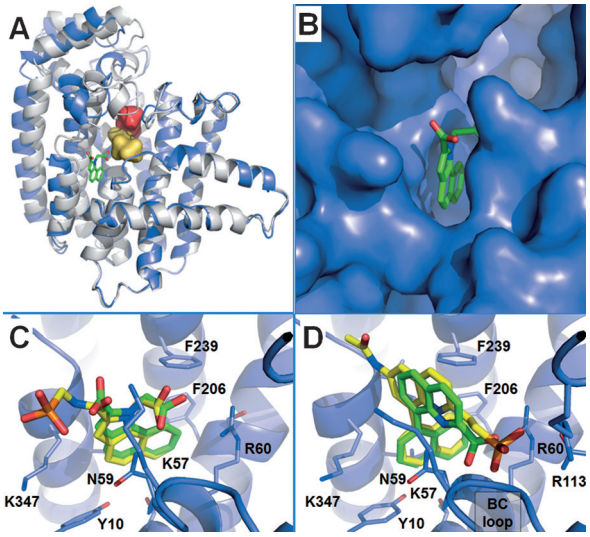

Figure 2. Crystal structure of the binary complex of FPPS with the allosteric inhibitor **6**.[9] A) Overall view of one FPPS subunit showing the location of the allosteric site (green stick model) in relation to the DMAPP/ZOL (red) and IPP (yellow) binding sites. Note the different conformational states adopted by the enzyme in the binary complex with **6** (open state, blue ribbon) and in the ternary complex with ZOL and IPP (close state, gray ribbon) B) Close-up view of the allosteric site in surface representation, showing the solvent accessibility of the carboxylic acid function in position 2 of the benzoindole tricyclic ring system. C) Close-up view of the complex with **7** (yellow), a phosphonate derivative of **6**, and structural overlay with **6** (green stick model). D) Close-up view (same orientation as (C)) of the complex with **15** (yellow), a phosphonate derivative of **13**, and structural overlay with **13** (green stick model).

allosteric FPPS inhibitors.[9b,14] In the salicylic acid series, **10** (the phosphonate analogue of **9**) was less potent (IC_{50} = 0.52 μM) than its parent compound (0.021 μM) but nevertheless showed submicromolar inhibition of FPPS. In the quinoline series, however, the vinyl phosphonate **14** (IC_{50} = 0.04 μM) was significantly more potent than the parent carboxylate analogue **13** (IC_{50} = 1 μM). Thus, for each of the three lead series, we could identify phosphonate analogues exhibiting submicromolar inhibitory activity (Scheme 1). X-ray analysis of the FPPS complexes with **7**, **13**, and **15**, a close analogue of **14**, confirmed that the phosphonate analogues adopt a very similar binding mode to that of their parent carboxylate, and explained the observed effects on the inhibitory potency. In the benzoindole series, the phosphonate moiety is highly exposed to bulk solvent and is further (4.7–5.1 Å) from the side-chain amino group of Lys57 than its carboxylate counterpart (4.0–4.3 Å), thus explaining the marginal reduction in inhibitory potency (Figure 2C). In the quinoline series, the vinyl phosphonate group points towards the binding site of the β-phosphate of IPP and is involved in multiple H-bonded contacts with the BC loop of the enzyme, as well as in strong, buried electrostatic interactions with Arg60 and Arg113, which contributes to significantly enhanced potency (Figure 2D and Figure S4). As shown by X-ray analyses,[9b] the binding modes of quinoline and salicylic acid derivatives largely overlap, with the carboxylic acid groups pointing in the same direction. Therefore, and in contrast to the benzoindole series, the conversion of these quinoline and salicylic acid derivatives into phosphonate analogues has potentially more impact on the inhibitory potency, as examplified here with **9**/**10** and **13**/**14**. Taken together, the allosteric pocket of FPPS can accommodate monophosphonate ligands with high affinity, as also recently shown by Tsantrizos and co-workers.[15]

While an in-depth investigation of the structure–activity relationships (SAR) of phosphonate derivatives for allosteric inhibition of FPPS would be needed for their further chemical elaboration and optimization, a solid understanding of the SAR for bone binding is equally important if this functionality is intended as a bone-targeting tag, so we shall focus on the latter. Figure 3 presents spectra for NMR-detected HAP binding for a series of salicylic acid derivatives (**9**–**12**; see Scheme 1 for structures). Figure 3A shows that the parent carboxylate compound **9** lacks bone affinity, which is in line with our initial observation with the AMP397 carboxylic acid analogue **5**. If a phosphonate functionality is attached directly to the aromatic ring system, as in compound **10** (Figure 3B), bone binding is still not detected. By contrast, when a single methylene spacer is introduced, as in **11** (Figure 3C), bone binding is clearly observed. Interestingly, bone affinity becomes even stronger when the linker is further elongated and thus made more flexible (**12**, Figure 3D). A qualitatively similar SAR for bone binding was observed in the benzoin-

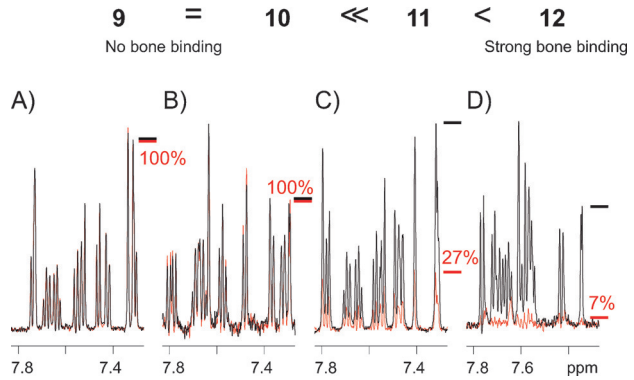

Figure 3. Affinity to hydroxyapatite (HAP) measured for several members of the salicylic acid series by using the NMR-based direct HAP binding assay.[10] The original allosteric FPPS inhibitor **9** showed no binding affinity to HAP (A), and nor did **10**, which has a phosphonate group directly replacing the carboxylic acid (B). However, as the length of the spacer between the aromatic system and the monophosphonate was increased and the monophosphonate-bearing substituent became more flexible, bone binding was enhanced (C, D).

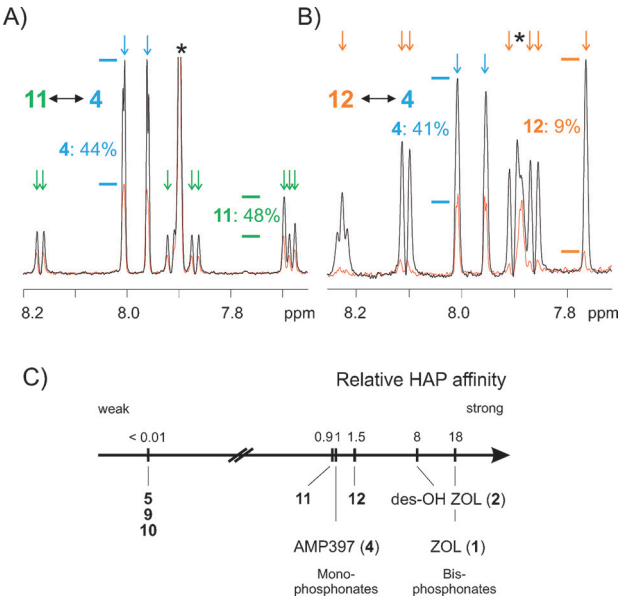

Figure 4. Relative affinities to HAP. A, B) Determination by NMR-based HAP binding assay of the relative affinities of monophosphonates **11** (A, green) and **12** (B, orange) in a competition format with AMP397 **4** (blue). Resonances of **4**, **11**, and **12** are marked by arrows in the respective color. The asterisks indicate an impurity. C) A graph depicting the relative affinities of the investigated compounds on a logarithmic scale.

dole series with **6**, **7**, and **8** (Figure S2). These data show that the presence of a phosphonate moiety alone is not sufficient to confer bone affinity to organic compounds. Furthermore, they show that the degree of bone affinity can be tuned by changing the length of the spacer, thus indicating that bone or HAP binding is limited by steric hindrance. While a single-atom spacer may provide sufficient flexibility to allow binding, affinity can be enhanced by using longer, two- to three-atom spacers. Interestingly, the vinyl phosphonic acid derivative **14** did not bind to HAP, thus further underscoring the requirement for flexibility when strong bone binding is desired. It may thus be possible to adjust bone affinity to the desired degree by varying both the length and chemical nature of the linker to the phosphonic acid group.

Preclinical in vivo data are available for AMP397 (**4**), which show that this compound accumulates in the skeleton as seen by quantitative whole-body autoradioluminography 72 h after AMP397 administration (Figure S1). It is thus interesting to compare the relative HAP affinities of AMP397 and our best-binding allosteric FPPS inhibitors. To this end, **11** and **12**, the members of the salicylic acid series with affinity to HAP, were subjected to the HAP binding assay in competition with AMP397 (**4**). As seen in Figure 4A, **11** and AMP397 have almost equal affinity to HAP (52% bound **11** versus 56% bound **4**). By contrast, **12** (which showed stronger bone binding than **11** in the direct-binding format, Figure 3D) clearly binds to HAP with higher affinity than AMP397 (Figure 4B: 91% bound **12** versus 51% bound **4**). This result is important because it correlates in vitro bone binding with in vivo bone accumulation data and allows us to predict the in vivo bone accumulation of monophosphonate FPPS inhibitors such as **11** and **12**.

As a summary of our investigations, the relative HAP affinities of the compounds discussed here are depicted in Figure 4C, which shows that monophosphonates such as **11**, **12**, or AMP397 can have an intermediate degree of HAP affinity compared to N-BPs such as ZOL. We have outlined herein a rational approach for conferring variable degrees of bone affinity on drug candidates. Our approach only requires the attachment of a phosphonate functionality to the drug molecule via a 1–3 atom spacer. This substituent can be attached to any suitable position that will not hamper binding to the target enzyme or receptor, as exemplified by the benzoindole series of FPPS inhibitors. Alternatively, as demonstrated with the quinoline series, it might be possible in favorable cases to confer some degree of bone affinity while simultaneously enhancing target binding. To this end, the recently developed NMR-based HAP binding assay is key for fast and robust assessment of bone affinity during lead optimization. While a more comprehensive analysis of the SAR for bone binding remains to be performed, our current data indicate that it is not very stringent, provided that the spacer is of sufficient length and flexibility. However, not every phosphonate moiety confers bone binding, and in fact, bone affinity has not been reported for phosphate- or phosphonate-containing prodrugs or metabolites. Still, medicinal chemists should have ample opportunities for tuning the desired degree of bone binding while maintaining potency and optimizing the overall physicochemical and pharmacological properties of the drug candidate.

The examples of the clinical compounds AMP397 and fosfomycin prove that monophosphonates can be orally available while exhibiting significant affinity to bone. The proposed strategy of attaching a phosphonic acid functionality as a bone-affinity tag can be useful for enhancing the safety and efficacy of antiresorptive or bone-anabolic drugs, such as inhibitors of c-Src,[16,17] cathepsin K,[18] colony stimulating factor-1 receptor (CSF-1R), selective androgen recep-

tor modulators (SARMs), selective estrogen receptor modulators (SERMs), or prostaglandin E2 conjugates.[19] The clinical development of such compounds is challenging because the respective targets occur not only in bone-resorbing or bone-forming cells such as osteoclasts or osteoblasts, but throughout the body, thus leading to undesired adverse effects. Targeting these modulators to bone thus has the potential to enhance tolerability and efficacy without compromising permeability or oral bioavailability. We hope that this approach will pave the way towards safer and more effective drugs for the treatment of bone diseases.

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