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Bisphosphonates: The role of chemistry in understanding their biological actions and structure-activity relationships, and new directions for their therapeutic use

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Abstract

The bisphosphonates ($(HO)_2P(O)CR^1R^2P(O)(OH)_2$, BPs) were first shown to inhibit bone resorption in the 1960s, but it was not until 30 years later that a detailed molecular understanding of the relationship between their varied chemical structures and biological activity was elucidated. In the 1990s and 2000s, several potent bisphosphonates containing nitrogen in their R^2 side chains (N-BPs) were approved for clinical use including alendronate, risedronate, ibandronate, and zoledronate. These are now mostly generic drugs and remain the leading therapies for several major bone-related diseases, including osteoporosis and skeletal-related events associated with bone metastases. The early development of chemistry in this area was largely empirical and only a few common structural features related to strong binding to calcium phosphate were clear. Attempts to further develop structure-activity relationships to explain more dramatic pharmacological differences *in vivo* at first appeared inconclusive, and evidence for mechanisms

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underlying cellular effects on osteoclasts and macrophages only emerged after many years of research. The breakthrough came when the intracellular actions on the osteoclast were first shown for the simpler bisphosphonates, *via* the *in vivo* formation of P-C-P derivatives of ATP. The synthesis and biological evaluation of a large number of nitrogen-containing bisphosphonates in the 1980s and 1990s led to the key discovery that the antiresorptive effects of these more complex analogs on osteoclasts result mostly from their potency as inhibitors of the enzyme farnesyl diphosphate synthase (FDPS/FPPS). This key branch-point enzyme in the mevalonate pathway of cholesterol biosynthesis is important for the generation of isoprenoid lipids that are utilized for the post-translational modification of small GTP-binding proteins essential for osteoclast function. Since then, it has become even more clear that the overall pharmacological effects of individual bisphosphonates on bone depend upon two key properties: the affinity for bone mineral and inhibitory effects on biochemical targets within bone cells, in particular FDPS. Detailed enzyme–ligand crystal structure analysis began in the early 2000s and advances in our understanding of the structure-activity relationships, based on interactions with this target within the mevalonate pathway and related enzymes in osteoclasts and other cells have continued to be the focus of research efforts to this day. In addition, while many members of the bisphosphonate drug class share common properties, now it is more clear that chemical modifications to create variations in these properties may allow customization of BPs for different uses.

Thus, as the appreciation for new potential opportunities with this drug class grows, new chemistry to allow ready access to an ever-widening variety of bisphosphonates continues to be developed. Potential new uses of the calcium phosphate binding mechanism of bisphosphonates for the targeting of other drugs to the skeleton, and effects discovered on other cellular targets, even at non-skeletal sites, continue to intrigue scientists in this research field.

Keywords

Bisphosphonates; Farnesyl diphosphate synthase (FDPS/FPPS); Osteoclasts; Bone resorption; Hydroxyapatite; Bone targeting

1. Introduction

It is now over 50 years since the biological effects of the bisphosphonates (BPs), originally called diphosphonates, were first reported in Science and Nature in 1969 [1].

The discovery and development of BPs as a major class of drugs for the treatment of bone diseases is a paradigm of a successful journey from ‘bench to bedside’. The study of BPs has become a vast field and more than 33,000 papers are currently listed on PubMed. In this article we will review the relationships between chemistry and pharmacological activity, building upon our publication from a decade ago [2]. We will also discuss advances in our current understanding of the structure-activity relationships with this drug class and look at what the future may hold.

The earliest uses of BPs were for bone scintigraphy and dental applications. The serendipitous discovery that BPs could inhibit bone resorption led initially to their first clinical approval for use in Paget’s disease. Later studies extended their use to the prevention

of skeletal-related events in patients with bone metastases, and to reduce fractures in patients with osteoporosis. Currently, BPs continue to be the leading medicines used for such disorders in which bone resorption is excessive.

A major landmark event in the history of BPs was the elucidation of how bisphosphonates work. The pharmacological effects of BPs as inhibitors of bone resorption appear to depend upon two key properties; their affinity for bone minerals, and their inhibitory effects on osteoclasts. Despite many attempts, it was not until the later 1990s that their detailed molecular mechanisms of action were elucidated [3].

Bisphosphonates are internalized by osteoclasts and interfere with specific biochemical processes. Bisphosphonates can be classified into at least two groups with different molecular modes of action. The simpler non-nitrogen containing bisphosphonates (such as etidronate and clodronate, Fig. 1) can be metabolically incorporated into non-hydrolysable analogues of ATP, which interfere with ATP-dependent intracellular pathways. The more potent, nitrogen-containing bisphosphonates (including pamidronate, alendronate, risedronate, ibandronate, minodronate, and zoledronate, Fig. 1) are not metabolised in this way but inhibit key enzymes of the mevalonate/cholesterol biosynthetic pathway. The major enzyme target for bisphosphonates is farnesyl diphosphate synthase (FDPS), and the elucidation of the crystal structure for this enzyme revealed how BPs bind to and inhibit at the active site *via* their critical N atom. Inhibition of FDPS prevents the biosynthesis of isoprenoid compounds (notably farnesyl diphosphate and geranylgeranyl diphosphate) that are essential for the post-translational prenylation of small GTP-binding proteins (which are also GTPases) such as rab, ras, rho, and rac, which are critical for intracellular signaling events within osteoclasts. These mechanistic advances now offer medicinal chemists and biochemists a more rational basis to explain the wide variations in pharmacological activity of structurally different BPs. The nitrogen-containing BPs (N-BPs) approved by regulatory agencies have now largely become generic drugs, as key patents have expired. Nevertheless, the bisphosphonates are likely to remain major drugs for treating bone resorptive diseases [4,5]. Innovation to improve oral absorption and other routes of delivery of the bisphosphonate drug class continues to be a goal, as their highly hydrophilic nature and metal chelation properties result in limited passage through lipophilic membranes.

There are emerging opportunities for extending the use of BPs to other areas of medicine, and an encouraging amount of research in this field has continued during the past decade. In particular several recent studies indicate that BPs may be associated with exciting and hitherto unexpected clinical benefits outside the field of bone disease, *e.g.* in increasing lifespan, reducing cardiovascular disease, and the reduction of several cancers. The range of non-skeletal effects of BPs also includes the inhibition of several protozoan parasites, immunomodulatory effects *via* monocytes/macrophages and T cells, increasing longevity in animal progeria models, and enhancing human stem cell lifespan, DNA repair, and tissue regeneration. Another area of active research is the development of BP conjugates for selectively delivering other drugs to the bone micro-environment. Promising results have already been achieved with anti-myeloma agents, antimicrobials, and epigenetic modulators [6].

2. Advances in understanding of structure-activity relationships of bisphosphonates

2.1. Update on the chemistry of bisphosphonates

Significant advances have been made in the chemistry of bisphosphonates, including improved synthetic methods and the design of new bisphosphonate molecules, which have enhanced the understanding of the mechanism of action and access to customized analogs with potentially new applications. Several reviews have described the chemistry of the current leading BPs used globally in clinical therapy (clodronate (CLO), alendronate (ALE), pamidronate (PAM), risedronate (RIS), and zoledronate (ZOL)) (Fig. 1) [2]. More recently, additional reviews of the specific clinically important BPs have appeared, providing even more details of their discovery and commercialization history (CLO: [7]; ALE: [8]; PAM: [9]; RIS: [10]; ZOL: [11]; Etidronate (ETI): [12]; Minodronate (MIN): [13]). In addition, multiple reviews regarding other general chemistry advances have also been published in the last decade, covering the topic from various perspectives. For example, several comprehensive reviews of recent developments in the chemical synthesis of new bisphosphonates with potential utility in bone metabolism-related diseases include those by McKenna et al. [14] and other researchers [15–18].

The “classical” method for the preparation of 1-hydroxymethylene-bisphosphonates, by which the more recent clinically utilized bisphosphonates are synthesized, involves some variation of heating an appropriate carboxylic acid with phosphorous acid and phosphorus trichloride, followed by hydrolysis to obtain the product, and the reaction is usually slow, requiring one or more days to complete the process [2]. Thus, one of the major challenges in the synthesis of BPs is the efficiency of these methods in manufacturing BPs for pharmaceutical uses on a large scale. Therefore, more recently the focus of many researchers has been on developing efficient, cost-effective, and green methodologies that are scalable, reduce the reaction time, and eliminate the use of hazardous solvents. Insightful review articles have continued to be published that have focused on new synthetic routes of these pharmaceutically valuable compounds. In a review article by Barbosa et al. [17], these key synthetic procedures, along with their limitations have been discussed in more detail for the process chemistry of the hydroxy BPs currently used pharmaceutically.

As also discussed previously [2], in this “classical” synthetic method to prepare geminal hydroxy BPs, the intermediate reaction product from the phosphonylation step is not homogeneous and tends to solidify. Thus continuous efforts with different solvents, including methanesulfonic acid [19–21], sulfolane [22,23], and ionic liquids [23–25], have been tried to improve the process. Also, because of the slow reaction time of these procedures, microwave irradiation offers a promising remedy and has been shown to be effective in up to prep-scale syntheses of several well-known bisphosphonates, while generating comparable yields in shorter reaction time [16,26,27].

Other new synthetic methods have continued to appear, representing a burgeoning interest in bisphosphonate drugs and the concomitant desirability of expanding the structural scope of this class. In the past decade, a number of new methodologies have been developed that

have given access to a wider library of BPs. Recently developed methodologies have granted chemists better control over the functionalization of BPs, as demonstrated in the following examples.

Substituting the α-hydroxy group for a different chemical moiety can dramatically change both enzyme inhibitory properties and mineral binding properties. Lawson, et al. [28] reported a new minodronate based bisphosphonate where the α-hydroxy group on the bisphosphonate bearing (geminal) carbon has been replaced with fluorine, 1-fluoro-2-(imidazo-[1,2 alpha]pyridin-3-yl)-ethyl-bisphosphonate, OX14 (**10**), through a useful fluorination sequence (Fig. 2). This resulted in the development of a strong inhibitor of FDPS, but with a lower binding affinity for bone mineral than most of the commonly studied bisphosphonates, as evidenced by both *in vitro* and *in vivo* experiments. This made OX14 (**10**) an interesting candidate for further investigation and potential development for its skeletal and nonskeletal benefits.

Other substitutions for the alpha hydroxyl of minodronate led to much reduced inhibition of FDPS. Mazur, Ebetino and colleagues also prepared some closely related heterocycles to the imidazopyridine in OX14, which have also been found to be potent FDPS inhibitors such as OX139 (Table 1) [29]. Alkyl substituents added to risedronate (**5**) or deshydroxy risedronate (**5a**), such as in OX78 (**5e**), OX104 (**5f**), OX105 (**5g**), OX111 (**5h**), and OX112 (**5i**), led to interesting potency enhancements (unpublished), which could be anticipated as these were shown with molecular modelling to overlap well with the hydrophobic pyridyl ring of minodronate [30,31]. However, attempts to utilize fluorine substituents were less successful for the risedronate ring than with the minodronate ring system presumably because the binding of the geminal hydroxyl is relied on more greatly with less alternate hydrophobic interactions in the FDPS site as with minodronate [32]. Phosphonocarboxylate modifications of both series, where one phosphonate is replaced by one carboxylate (**5d** and **7d**), led to analogs with little FDPS and no GGPPS inhibitory properties, but an emergence of inhibition of the RAB GGTase enzyme was observed [33–35]!

In 2017, Engelsma et al. reported a protocol that enables the controlled synthesis of mono- and diester methylene bisphosphonates [36]. They developed a new class of orthogonally protected phosphonylmethylphosphonate reagents (**11**) through treating a lithiated methylphosphonate diester with a chlorophosphoramidite (Fig. 2). Subsequently, **11** can be coupled to a variety of alcohols through a P^{III} phosphoramidite coupling/oxidation. Deprotection of **12** at this step would enable the preparation of terminally substituted BPs, while treating **12** with secondary alcohol through phosphodiester condensation would lead to a precursor of unsymmetric BPs (**13**). This protocol facilitates the introduction of BPs in structurally diverse pyrophosphate-containing biomolecules and is particularly advantageous for the development of BP analogues of natural products (ATP, NAD, FAD).

Egorov et al. have successfully developed a simple method for the synthesis of (geminal) α-hydroxy bisphosphonates (HBPs) (**14**) through a one-pot reaction of carboxylic acids and tris(trimethylsilyl) phosphite in the presence of catecholborane (Scheme 1) [37]. This method allows access to a larger library of HBPs, given that there are so many carboxylic acids commercially available. Moreover, this method is particularly useful for the synthesis

of the highly useful nitrogen-containing hydroxy bisphosphonates, as it moves away from the additional steps often necessary for the protection/deprotection of the amino moieties.

Zhang et al. have developed an efficient methodology that creates structurally diverse aromatic bisphosphonates (**15**) through an Rh(III)-catalyzed directed methylene-diphosphonate carbenoid insertion into aromatic C—H bonds (Scheme 2). This one-step reaction combats the limitations of the traditional methods for the synthesis of aromatic bisphosphonates as they suffer from limited functional group diversity [38].

Another highly valuable synthetic methodology that has been developed in recent years is for the synthesis of α-, β-, and γ-azido bisphosphonates. The introduction of the azido moiety has created a new and useful bisphosphonate synthon, as it can readily and efficiently engage in "click chemistry" [39,40]. The first examples of the synthesis of α-azido BPs (**16**, **17**) were reported by Chamberlain et al. in 2011 (Scheme 3a) [41]. This is an example of an electrophilic azido transfer, where tosyl azide reacts with a carbanion to generate a triazene intermediate, which fragments into diazo and azido products. Chamberlain et al. were able to obtain the azido compound as the major product by controlling the reaction conditions. Later in 2019, an article by Chiminazzo et al. reported an optimized protocol affording (**18**, **19**) β- and γ-azido bisphosphonates (Scheme 3b). Moreover, they have successfully conjugated these useful synthons to red-fluorescent diketopyrrolopyrrole by "click chemistry" to make a novel fluorescent probe for *in vitro* bone imaging [42].

New methodologies developed by Li et al. and Zhu et al. provide efficient access to a range of spiro bisphosphonates (Scheme 4) [43,44]. Through a 1,3-dipole cycloaddition reaction promoted by montmorillonite, Li et al. obtained spiro[indole-pyrrolizine], spiro[indole-indolizine], and spiro[indole-pyrrolidine] *gem*-bisphosphonates in good to excellent yields (Scheme 4a) [43]. Zhu et al. report a [3 + 2] cycloaddition reaction of azomethine imines with tetraethyl vinylidene BP catalyzed by CuI to furnish a series of dinitrogen-fused heterocycles coupled to geminal bisphosphonates (Scheme 4b) [44].

In the last decade, various "green" synthetic protocols have been developed to afford more facile access to α-substituted aminomethylene bisphosphonates (**22**). A straightforward synthesis of N-BPs consists of a one-pot reaction of diverse aryl/heteroaryl amines with dialkyl phosphite and triethyl orthoformate (Scheme 5a). Using conventional heating (oil bath) in these reactions would afford moderate yields of the corresponding α-amino bisphosphonates, and completion of the reaction generally takes a few hours [45,46]. Recently, by using microwave irradiation, eco-friendly protocols with comparable yields and shorter reaction time have been achieved (Scheme 5b) [26,47,48]. Employment of microwave irradiation in conjunction with the use of catalysts such as CuO nanoparticles [49], ZnO nanoparticles [50], and rGO-SO_3H [51] have provided highly efficient green protocols that give access to a wide scope of α-amino bisphosphonates (Scheme 5c). Given the high demand for α-amino bisphosphonates in medicinal chemistry [52], such green and high yielding methodologies would facilitate the manufacturing of these pharmaceutically valuable motifs. Some recently synthesized α-amino bisphosphonates have been tested as antioxidant, antimicrobial, and anti-cancer drugs, and applications of such BPs will be discussed later in this review.

It is noteworthy that BPs are also used as synthons in the preparation of nucleotide analogs. When such BP analogues have different substituents at the bridging bisphosphonate carbon (as is the case with all the N-BP drugs), that carbon becomes chiral due to the asymmetric phosphonate groups in the nucleotide analogues. In a recent report, McKenna and collaborators have created a series of chiral synthons derived from differently substituted α-halo BPs which can be used to prepare individual stereoisomers of nucleotide BP analogues that showed non-equivalent interactions with an enzyme binding site, depending on the absolute configuration at the bridging carbon (Scheme 6) [53].

2.2. Bisphosphonate esters and prodrugs

2.2.1. Bisphosphonate esters—Bisphosphonate esters are an interesting group of bisphosphonate derivatives, particularly those that have unique pharmacological properties of their own, that differ from the bone effects of classical bisphosphonates.

In the context of seeking potentially useful bisphosphonate derivatives, esters offer some novel properties. Being lipid soluble, they should have better absorption from the gut. Labile esters might also be used as prodrugs with improved bioavailability.

The compound called Apomine (not to be confused with Apomorphine^{R}) is a 1,1-bisphosphonate ester (Fig. 3) which has been studied in considerable detail for its unique pharmacological properties.

Apomine was one of several such compounds discovered by Eric Niesor and Craig Bentzen and colleagues at the Symphar SA company in Switzerland [54,55], and became the most intensively studied for its potential hypocholesterolemic [56–58] and antineoplastic activities [59].

The pioneering studies of this group showed that 1,1-bisphosphonate esters had the unexpected property of inducing hypocholesterolemia in experimental animals, an effect that was attributed to increasing the rate of degradation of HMGCoA reductase, a key enzyme in the cholesterol biosynthetic pathway and the well-known site of action of statin drugs. The mechanism underlying this hypocholesterolemic effect appears to be due to the ability of these esters to activate the orphan nuclear receptors FXR and LXR alpha. The farnesoid X activated receptor (FXR) is a member of the nuclear hormone superfamily implicated in cholesterol metabolism and bile acid transport. Natural endogenous agonists of FXR, like farnesol, as well as synthetic FXR agonists, like apomine, trigger differentiation, inhibit cell proliferation and are potent inducers of apoptosis. These actions may also contribute to their antineoplastic effects. These early studies with apomine (SR-45023A) on cholesterol metabolism led to it being studied by SmithKline Beecham as a novel cholesterol-lowering agent.

Apomine also underwent quite extensive development as an antineoplastic drug [59]. Preclinical studies showed activity against various tumor cell types, including osteosarcoma [60], myeloma [61,62], melanoma [63], and breast cancer [64]. Various clinical oncology studies were conducted, initially by Ilex Oncology based in San Antonio, which was later acquired by Genzyme Oncology in 2004. The clinical studies reached phase 2 exploration

and included studies in refractory melanoma [65] and various solid tumors, including ovarian and prostate cancers. Despite several clinical studies and exploratory trials, apomine never reached the status of an approved drug however.

Pharmacologically, apomine is orally bioavailable but is a stable ester, so does not undergo cleavage and exhibit the typical bone effects of bisphosphonates. Pharmacokinetic studies were conducted in normal subjects as well as in cancer patients [66]. We might wonder whether the early promise apomine offered as a therapeutic with unique properties was thwarted by successive company mergers and acquisitions. Glaxo Wellcome merged with SmithKline Beecham in 2000 to become GlaxoSmithKline plc (GSK), and Sanofi acquired Genzyme in 2011. There are many examples of interesting drugs being victims of changing priorities when companies change hands and when key inventors are no longer able to champion their discoveries.

Nonetheless, the orphan nuclear receptors FXR and LXR alpha continue to be targets of interest for the discovery of new therapeutic agents. Bile acids and hydroxysterol intermediates are the respective natural ligands of these two structurally and functionally closely related receptors. Both FXR and LXR alpha are thought to play a major role in the control of cholesterol catabolism by regulating the expression of cholesterol 7-alpha-hydroxylase, the rate limiting enzyme of bile acid synthesis in the liver. Upon ligand binding, FXR regulates key genes involved in the metabolic processes of bile acid synthesis, transport and reabsorption and is also involved in the metabolism of carbohydrates and lipids. FXR is therefore considered to be a promising drug target for the therapy of bile acid-related liver diseases. With the approval of obeticholic acid (OCA) as the first small molecule to target FXR, several other small molecules are being evaluated in clinical trials. These newly emerging therapies based on agonists and antagonist of FXR are described in a recent review [67]. Interestingly, apomine is not specifically mentioned. However, apomine was recently identified as one of several compounds that might be repurposed for anti-viral activity against Covid infections [68].

As reviewed previously [69], bisphosphonate esters were also studied for their pharmacological effects useful in animal models of inflammation and arthritis. Nugent and coworkers reported studies to establish these effects with analogs related to pyrazoline (Formula A, Fig. 3), however, no compounds from this series ever advanced to clinical development [70]. It is uncertain whether these are *in vivo* prodrugs of the corresponding phosphonic acids.

2.2.2. Prodrugs—Bisphosphonates are notorious for their hydrophilic nature. This contributes to their excellent soft tissue safety as transport through many cell types and soft tissues is limited. Low oral absorption, and almost no absorption, reputedly due to an insoluble calcium salt formation “food effect” is also associated with orally administered bisphosphonates. While there are extensive studies on the phosphate and phosphonate prodrugs (reviewed in [71–73]), our focus here will be solely for studies of prodrugs of clinically used bisphosphonates. A group at P&G did demonstrate that addition of calcium chelators such as EDTA to oral formulations reduces the loss of absorption due to food intake prior to oral administration and the product Atelvia® was marketed as an any time

formulation of risedronate that eliminated the need for fasting prior to weekly dosing of risedronate (Actonel®) [74]. Other researchers have investigated various ester, peptide (amide linkage) and anhydride prodrugs and formulations (for etidronate, clodronate, pamidronate and alendronate) for enhanced uptake after oral dosing and the studies have been reviewed extensively [75,76]. Those prodrug design strategies focus on masking the phosphonate groups as well as the –OH group on the bridging carbon and $–NH_2$ group of the side chain substituent (Scheme 7). Webster et al. also evaluated the phosphonamidate prodrug of clodronate (compound **2a**), and found the significant enhancement of *in vitro* anticancer activity of the prodrug compared to clodronate [77]. It should be noted that for the purpose of increasing oral bioavailability for bone disease treatment, BP prodrugs should have enough lability to be able to efficiently release the parent drugs in the bloodstream, but maintaining sufficient GI stability. In the case of BP prodrugs useful for extraskeletal diseases (*e.g.*, cancer), it may be favorable for the prodrugs to have a somewhat slower cleavage in plasma to reduce the rate of sequestration on bone to allow greater distribution to other tissues. Additionally non-clinical studies have been reported demonstrating the potential utility of certain cyclic acetal esters of several nitrogen-containing bisphosphonates (*e.g.* risedronate ester (**5j**)). Although this series was not designed to enhance oral absorption in the fasted state, they appeared to be stable enough in the GI tract to dramatically reduce the food effect on orally administered bisphosphonates and then readily hydrolyze to the parent bisphosphonate (risedronate (**5**)) in the bloodstream (Scheme 8) [78].

2.3. Farnesyl diphosphate synthase (FDPS) inhibition: the key enzymatic target of nitrogen-containing bisphosphonates in osteoclasts – an interesting journey

The current understanding of the mechanisms of actions of BPs was not fully elucidated for almost thirty years after their first successful clinical use. The discovery of the major enzymatic target of N-BPs responsible for their antiresorptive activity has an interesting history and one not typical for modern drug development, where a clear identification of molecular targets and understanding of mechanisms of action is often required prior to regulatory approvals. The identification of the key enzymatic target, within the osteoclast, of these more recently developed, highly potent antiresorptive bisphosphonates (**5**–**9**) (Fig. 1) is perhaps the most important finding for this drug class since the discovery of the antiresorptive activity of BPs in the late 1960s. The key enzyme target, farnesyl diphosphate synthase (FDPS), was finally identified after the discovery of the involvement of the mevalonate pathway as a site of action of N-BPs. Followup studies using other research approaches, including protein crystallography, further illustrate the precise interactions between N-BPs and FDPS. The details of how bisphosphonates act within cells, and the details of this discovery history have been recently reviewed by Rogers et al. in the BP 50th anniversary celebratory issue of Bone [3]. The related *in vitro* assays based on inhibition of FDPS are now used as a semi-routine method to screen and evaluate the efficacy of newly synthesized BP molecules. Alongside the identification of FDPS, other enzymes in the mevalonate pathway, *e.g.*, geranylgeranyl diphosphate synthase (GGPPS), were also found to be additional targets of some new BP molecules, and efforts to study in more detail the utility of the molecular interactions of BPs and FDPS or other key enzymes in the mevalonate pathway from different species have continued to emerge in the last decade.

Interestingly, the clinically used N-BPs all have weaker activity against geranylgeranyl diphosphate synthase (GGPPS) than FDPS. GGPPS is the enzyme beyond FDPS in the mevalonate pathway. When this weaker inhibition of GGPPS was first discovered, it was perhaps a rather surprising finding since it appears that it is the reduction in the levels of the metabolite GGPP, rather than FDP, that explains how BPs inhibit osteoclast function, which is the basis for their use in clinical disorders of bone resorption. This is because the GTP-ase signaling proteins prenylated by GGPP are more critical to osteoclast function than those prenylated by FDP. The probable explanation of this apparent paradox is that inhibition of FDPS reduces the levels of both metabolites, FDP and GGPP. In many experimental studies, the addition of GGPP reverses the biological actions of N-BPs, confirming that these are the key enzymes critical for the effects of N-BPs on osteoclast function.

In a recent article by Manaswiyoungkul and coworkers, the inhibitory potency of bisphosphonates towards human FDPS (hFDPS) and human GGPPS (hGGPPS) has been again extensively reviewed [79]. Selected recent examples that particularly shed light on the complexities of FDPS binding and inhibition are discussed in the sections below.

2.4. Insights from the interactions of bisphosphonates and FDPS via protein crystallography

It is now known that the BPs bind to the allylic (dimethylallyl diphosphate (DMADP) or geranyl diphosphate (GDP)) binding site of FDPS *via* three Mg^{2+} ions and a network of water molecules. The binding site contains two aspartate-rich motifs (^{103}DDIMD^{107} and ^{243}DDYLD^{247}) which upon binding of BP produces a conformation change from open to partially closed. This creates a cavity for the binding of the homoallylic substrate *i.e.* isopentenyl pyrophosphate (IPP). Thus, the binding of IPP to FDPS rigidifies the C-terminal ^{350}KRRK^{353} loop which causes the enzyme to develop a closed conformation to lock the BP and IPP inside the protein. Our team [2], and more recently, Park et al. [80] have provided further details of the binding of various BPs to FDPS.

Enzyme mutants have also been generated and analysed to further substantiate the key binding interactions required for optimal inhibition [81]. This includes investigation of the role of threonine 201 (Thr201) and tyrosine 204 (Tyr204) residues in substrate binding, catalysis, and inhibition by N-BPs, where kinetic and crystallographic studies of mutated FDPS proteins were employed. Mutants of Thr201 illustrated the importance of its methyl group in aiding the formation of the isopentenyl pyrophosphate (IPP) binding site, while Tyr204 mutations revealed the previously unknown role of this residue in both catalysis and IPP binding. The interaction between Thr201 and the side-chain nitrogen of N-BP was shown to be important for tight binding inhibition by zoledronate (ZOL) and risedronate (RIS), and RIS was shown to interact with the main-chain carbonyl of Lys200. The interaction of RIS with the phenyl ring of Tyr204 proved essential for the maintenance of the isomerized enzyme-inhibitor complex. Studies with conformationally restricted analogues of RIS reaffirmed the importance of Thr201 in the formation of hydrogen bonds with N-BPs. Thus new features of FDPS inhibition by N-BPs have recently been elucidated and additional unknown roles of the active site residues in catalysis and substrate binding were revealed in these studies [81].

More work has continued to enhance the understanding of bisphosphonate binding characteristics of both FDPS and GGPPS. Notably, structure-activity studies from the Oldfield group, in related non-mammalian enzymes in particular, have led to the identification of additional bisphosphonates with exquisite sensitivity and specificity utilizing molecular modelling and crystallography techniques. The utility of this work has led to intriguing leads for parasitic diseases, anti-malarials [82], anti-tumor agents [83–85], and antibiotic research [86] (Fig. 4) (see detailed discussion in later sections in this manuscript).

2.4.1. Recent studies of the binding of BPs to human FDPS—Yokoyama et al. [87] used neutron crystallography to further describe the protonation states and hydration structure of risedronate bound to FDPS. They obtained data for FDPS-RIS and FDPS-RIS-IPP complexes (Fig. 5A). In both X-ray structures, the pyridyl nitrogen was protonated. The pyridyl nitrogen formed a bifurcated H-bond with the side chain of Thr201 and the main chain carbonyl of Lys200. This main chain carbonyl of Lys200 also formed a CH–O bond with the 4-H of pyridine. The phosphonate groups of risedronate were completely deprotonated. Eight aspartic acid residues (Asp 103, 104, 107, 174, 243, 244, 247, and 261), Gln171, and Lys 266 interact indirectly with the phosphonate groups *via* water molecules and Mg^{2+} ions, while three basic amino acids Arg112, Lys200, and Lys257 interact directly with the phosphonate groups. The IPP was found to be fully deprotonated and interacting with basic residues and Mg^{2+} ions within the binding site. Neutron crystallography also revealed that the water molecules in the binding site are neither hydronium nor hydroxide ions. Hence, the neutralization of charge in the binding site upon the binding of RIS occurs *via* protonation-deprotonation of the adjacent amino acids. Thus, protonation of Asp264 enhances the binding of risedronate in FDPS. This work demonstrated that the pyrophosphate group of IPP was fully deprotonated and binding of IPP leads to the protonation of Glu93. Glu93 and Asp264 play an important role in stabilizing the net charge in the binding site to further enhance inhibitory potency. Also, it was shown that the hydroxyl group at the central bisphosphonate bearing carbon of risedronate enhances Mg^{2+} binding, further establishing its importance to enzyme binding, as well as binding to hydroxyapatite mineral surfaces.

Leung et al. [88] synthesized a series of thienopyrimidine BPs which were potent inhibitors of hFDPS. An example from this series is **23** which was 8-fold less active than risedronate against hFDPS (IC_{50} 39 nM *vs* 5.2 nM). The crystal structure of **23** - hFDPS shows that this thienopyrimidine BP can bind to the allylic (DMADP/GDP) binding portion of the enzyme in a similar orientation to that of risedronate (Fig. 5B). Thus, the phosphonate moieties of **23** can form similar interactions as those of risedronate. However, in comparison to the bifurcated H-bond formed by the pyridyl nitrogen with the side-chain hydroxyl of Thr210 and the main chain carbonyl of Lys200, the N3 of the thienopyrimidine ring only interacts with the side chain hydroxyl of Thr210, possibly reducing somewhat its overall enzyme binding/inhibition as *in vitro* studies suggest. The aromatic side chain of **23** extends into the lipophilic portion of the binding site containing two phenyl groups (Asp98 and Asp99), Leu100, and side-chain carbons of Gln171. Furthermore, the cyclopropyl ring extends towards the dimer interface of the homodimeric protein.

In addition to occupying the allylic (DMADP/GDP) binding site, **23** also occupies portions of the homoallylic (IPP) binding site, thus making it impossible for the IPP to bind concomitantly. Instead, the crystal structure of **23** – hFDPS contains inorganic pyrophosphate which functions similarly to IPP in closing the FDPS binding site, although no prenylation can then occur. Unlike risedronate, **23** lacks the Cα-hydroxy group which is known to increase the binding affinity of N-BPs to bone. Nevertheless, **23** had higher bone affinity than risedronate based on NMR studies. The higher bone affinity of **23** despite the lack of the Cα-OH group may result from less calcium salt solubility due to increased lipophilicity, or binding contributions from the other polar groups in the structure. Although no *in vivo* proof of concept studies are reported, the significance of this modification appears to allow improved cellular uptake as evidenced in their *in vitro* inhibition studies on myeloma cells. This could have important *in vivo* implications as good FDPS and bone binding characteristics are maintained.

The same group also designed a series of 2- and 3-aminopyridine BPs that showed inhibition of hFDPS comparable to risedronate [89]. A comparison of the binding modes of the 2-amino and 3-aminopyridine BPs showed that the pyridyl nitrogen of the 2-aminopyridine (**24**) interacts with Gln240 *via* a water molecule but it does not interact directly with other residues (Fig. 5C, D). In comparison, the pyridyl nitrogen of the 3-aminopyridine (**25**) forms a bifurcated H-bond with the side chain of Thr201 and main chain carbonyl of Lys200 reminiscent of the binding of risedronate pyridine. Nevertheless, both 2 and 3-aminopyridine analogs showed comparable activity against hFDPS (IC_{50} = 12 nM and 16 nM respectively). Thermodynamic characterization of the binding of the 2- and 3-aminopyridine BPs to hFDPS suggests that the enthalpic gain attained by the 3-aminopyridine BP (**25**) from the bifurcated H-bond formation is negated by entropic loss in the lipophilic portion of the molecule when compared to the 2-aminopyridine BP (**24**). Such findings will further fuel the discovery of analogs with strong FDPS binding, but with differing physiochemical properties that may offer leads towards analogs with improved nonskeletal benefits as well.

2.4.2. Binding of BPs to non-mammalian FDPS—In efforts to facilitate the development of new antiparasitic agents, Aripirala et al. [90] studied the binding of three pyridinium BP analogs to *Leishmania major* FDPS (LmFDPS) using X-ray and ITC. All three compounds are bound to the allylic (DMADP/GDP) binding site of LmFDPS (Fig. 6). Compounds **27** and **28** had similar binding modes to N-BPs. The binding of **27** and **28** with the LmFDPS allylic binding site involved three cations and IPP. In case of **26**, there were two cations but no IPP. The authors suggest that the LmFDPS-**26** complex might be an intermediary structure and it supports the ordered binding theory where the allylic sites on the homodimeric protein are occupied first leading to the formation of asymmetric monomers where one monomer was more favored for IPP binding. Binding of IPP to the first monomer leads to conformational change in the second monomer thus facilitating the binding of the second IPP. ITC studies of zoledronate and the three pyridinium BPs binding to LmFDPS suggest that the process was entropy-driven. Zoledronate and **26** bound more tightly to the enzyme than **27** which had a less favorable enthalpic component. Nevertheless, **27** still had better inhibitory activity than the other ligands which may be attributed to the

more favorable entropic contribution arising from displacement of water by the phenyl ring from the hydrophobic region of the binding site.

Yang et al. [91] synthesized and tested a series of lipophilic BPs as potential antiparasitics for Trypanosomes. The series consisted of pyridinium, sulfonium, aminomethylene BPs, as well as N-alkyl imidazolium analogs of zoledronate and dehydoxyzoledronate. These were screened for biological activities in *T. brucei* FDPS enzyme inhibition, *T. brucei* cell growth inhibition, and also human cell (HEK293T) cytotoxicity assay in order to determine their selectivity index (SI = $HEK293T_{CC50}$/*T. brucei*$_{EC50}$). Compounds with chain lengths of zero to seven carbons (C_0-C_7) showed comparable inhibitory activities against TbFDPS. The activity of the compounds decreased if the chain length was more than nine carbons (C_9) which most likely was due to steric hindrance. Among the pyridiniums, sulfoniums, and aminomethylenes, the best compound was **29** (BPH-597) (TbFDPS IC_{50} = 0.25 ± 0.29 μM, SI = 4.3) which contained a six-carbon C_6 chain at the 3-position of the pyridine ring. Among the N-alkyl imidazolium series, the dehydroxyzoledronate analog **30b** (BPH-1326) (TbFDPS IC_{50} = 0.56 ± 0.13 μM, SI = 62) containing a seven-carbon chain and zoledronate analog **31b** (BPH-1238) (TbFDPS IC_{50} = 1.26 ± 0.6 μM, SI = 293) containing a nine-carbon chain displayed the best overall activities. X-ray studies showed that the binding mode of these compounds is similar to the N-BPs. They bound to the allylic (DMADP/GDP) binding site of TbFDPS *via* three Mg^{2+} ions and a network of water molecules (Fig. 7). Thermodynamic studies using ITC showed that the binding is strongly driven by entropy. The addition of the seven-carbon chain to dehydroxyzoledronate **30a** (BPH-301) led to an increase in the entropic (ΔS) value (53 cal mol^{-1} K^{-1} for **30b** *vs* 41 cal mol^{-1} K^{-1} for **30a**). This was a favorable entropic contribution as **30b** displayed better inhibitory activity against TbFDPS than **30a** (IC_{50} = 0.74 ± 0.7 μM, SI = 0.3). **30b** was 4-fold less active than zoledronate (IC_{50} = 0.14 ± 0.057 μM, SI = 0.5) against the TbFDPS but had better *T. brucei* cell inhibitory activity (EC_{50} = 1 ± 0.0078 μM *vs* 14 ± 1.5 μM) and displayed a better selectivity index.

2.4.3. Binding of lipophilic BPs to other non-mammalian enzymes—Malwal et al. [86] designed lipophilic BPs that inhibit bacterial growth by targeting bacterial cell wall and quinone biosynthesis (Fig. 4). These BPs target the long-chain prenyltransferase enzyme octaprenyl diphosphate synthase of *E. coli* (EcOPPS) and the corresponding heptaprenyl diphosphate synthase of *S. aureus* (SaHepPPS). In addition, these BPs also target the FDPS enzyme due to the structural similarity of the active sites of these enzymes. Initial screening of a diverse set of compounds identified the long alkyl chain pyridinium analog **32** (BPH-715) as a potent inhibitor of both SaHepPPS (IC_{50} = 110 nM) and EcOPPS (IC_{50} = ~6 nM). **32** was previously reported to be a potent inhibitor of tumor cell growth with activity against both FDPS (IC_{50} = 100 nM) and GGPPS (IC_{50} = 280 nM). When screened against a panel of gram-positive and gram-negative bacteria, **32** was only active against gram-positive bacteria. Thus, **32** displayed good activity against *S. aureus* (ED_{50} = 10 ± 0.68 μg/mL) but it had weak or no activity (ED_{50} > 21 μg/mL) against *E. coli*. When compared with other structures in their set, compounds containing an aromatic group at the end of the lipophilic chain were generally more active against gram-negative bacteria. For example, compound **33** (BPH-1625) had quite good activity against gram-negative bacteria (ED_{50} =

~2 μg/mL) but it had weak activity against gram-positive bacteria (ED_{50} > 30 μg/mL). Such information will be valuable when developing selective inhibitors against gram-positive and gram-negative bacteria. SAR study of **32** led to compound **34** (BPH-728), which had comparable activity to **32** against SaHepPPS (IC_{50} = 100 nM), EcOPPS (IC_{50} = 12 nM) and human FDPS (IC_{50} = 230 nM).

The authors were unable to obtain an X-ray structure of their most active inhibitors bound to SaHepPPS or EcOPPS. However, they were able to obtain the structure of a weaker inhibitor **35** (BPH-629, IC_{50} = 330 μM) bound to EcOPPS (Fig. 8). Compound **35** bound to the allylic (farnesyl diphosphate, FDP) binding site of EcOPPS and coordinated with only one Mg^{2+} ion. Arg93 and Asp88 directly interacted with one of the phosphate groups while Asp84 interacted with the second phosphate *via* Mg^{2+} ion. The phenyl ring formed hydrophobic interactions with Thr80 while the tricyclic ring formed hydrophobic interactions with side-chains of Leu110, Ile142, and Ala143, and the side-chain carbons of Glu146 and Asp113. Compound **34** is expected to bind in a similar fashion as **35**.

2.5. Further studies of interactions between bisphosphonates and GGPPS via protein crystallography

As noted earlier, the clinically used N-BPs have weaker activity against geranylgeranyl diphosphate synthase (GGPPS) than FDPS. GGPPS is the enzyme succeeding FDPS in the mevalonate pathway.

GGPPS catalyzes the formation of geranylgeranyl pyrophosphate (GGPP, C_{20}) by condensing farnesyl diphosphate (FDP, C_{15}) and isopentenyl pyrophosphate (IPP, C_5). The catalytic site of GGPPS contains two aspartate-rich motifs similar to FDPS and the catalytic mechanism of the two enzymes is expected to be similar. The catalytic site of GGPPS is larger than FDPS in order to accommodate the larger GGPP product. Unlike the human FDPS enzyme which exists as a homodimer, the human GGPPS (hGGPPS) is a complex hexameric propeller-blade shaped structure consisting of three homodimers which makes it difficult to crystallize and study hGGPPS – inhibitor interactions. Most of the current knowledge regarding GGPPS – BP interactions comes from yeast GGPPS (yGGPPS) – BP X-ray structures. In comparison to hGGPPS, the yGGPPS exists as a homodimer and the BPs bind to the allylic binding site *via* Mg^{2+} ions and water molecules. Unlike FDPS where three Mg^{2+} are required to bind to BP, the number of Mg^{2+} ions in the binding site of yGGPPS - BP X-ray structures varies from zero to three. Recently, the first structure of hGGPPS complexed with BP (ibandronate) was reported [92]. Ibandronate (**9**) bound the allylic (FDP) binding site of GGPPS with the bisphosphonate moiety interacting with the aspartate-rich regions (^{64}DDIED^{68} and ^{188}DDYAN^{192}) *via* three Mg^{2+} ions and a network of water molecules (Fig. 9A). The three Mg^{2+} ions were found to be crucial for hGGPPS – ibandronate interaction using ITC experiments and computational studies. Based on this finding, the authors reanalyzed several of the yGGPPS – BP X-ray structures and were able to assign three Mg^{2+} ions in the binding site which improved the overall quality of the X-ray structures. The X-ray structure of hGGPPS – ibandronate (**9**) will be a valuable tool for finding new GGPPS inhibitors using computational methods.

The same research group also reported the structure of mutant GGPPS-D118Y in complex with zoledronate (**6**) [93] (Fig. 9B). The D118Y mutation in GGPPS has been associated with increased susceptibility to BP-induced atypical fractures [94,95]. The GGPPS-D188Y mutant was ~4-fold less active than the wild-type GGPPS and had a reduced affinity for zoledronate. X-ray structure of the GGPPS-D188Y – zoledronate complex shows the displacement of Tyr188 compared to the apo structure to accommodate zoledronate (**6**) and one Mg^{2+} ion. Nevertheless, zoledronate (**6**) significantly reduced the activity of GGPPS-D188Y at physiologically relevant concentrations. It is noteworthy that while the residual activity of the wild-type GGPPS after zoledronate inhibition may be sufficient to carry out critical bone functions, inhibition of the GGPPS-D118Y mutant which already has low enzymatic activity may result in impairment in bone remodeling. Since this mutation was found in a family of three sisters with osteoporosis [94,95], it is still unclear whether it has a causal and wider relationship to the occurrence of atypical fractures associated with BP administration. Nonetheless these findings indicate a potentially interesting new direction of BP drug development by exploiting novel BPs that target hFDPS, but are less active on hGGPPS than the ones currently available, with the aim of reducing the side-effects of BPs used to treat bone diseases.

Inhibitors of hGGPPS may have potential use in the treatment of different cancers such as colorectal cancer [83], metastatic prostate cancer [84], and lung adenocarcinomas [85]. The majority of the BP-based inhibitors of GGPPS are characterized by one or two lipophilic chains in their structure. One of the most potent and selective hGGPPS inhibitors is a triazole-based BP (**36**) which has IC_{50} of 45 nM and is >600-fold selective for hGGPPS *vs* hFDPS [96].

Another example is the C2-thienopyrimidine BP compound **37a** which has IC_{50} of 42 nM and is >24-fold more selective for hGGPPS *vs* hFDPS. The C2-thienopyrimidine BPs block the proliferation of multiple myeloma (MM) cells by inhibiting protein prenylation. In order to study the binding of **37a** to hGGPPS, the authors generated an easily crystallizable version of hGGPPS *via* Y246D mutation in the protein (Fig. 9C). The hGGPPS – Y246D mutant was dimeric in nature and was catalytically active. A low resolution hGGPPS(Y246D) – **37a** complex showed that the compound bound to the allylic (FDP) binding site with the *p*-fluorophenyl extending into the hydrophobic cavity that would accommodate the farnesyl group of FDP. The low resolution of the X-ray structure hampered the assignment of the Mg^{2+} ions, however the requirement for Mg^{2+} ions was confirmed by differential scanning fluorimetry thermal shift assays. Another C2-thienopyrimidine BP from the series, compound **37b** which has an IC_{50} of 86 nM and is ~16-fold more selective for hGGPPS *vs* hFDPS, displayed antimyeloma effects in a MM disease mouse model thus validating hGGPPS as therapeutic target for MM.

Furthermore, as discussed in our previous review [2], the BP-based inhibitors of GGPPS were exemplified by the compound digeranyl bisphosphonate **38** (DGBP, Fig. 10A), synthesized by the Wiemer group in 2007. Interestingly, this compound was crystallized with yeast GGPPS by the Oldfield group as part of their crystallographic studies of BP inhibitors of this enzyme [97]. As can be seen (Fig. 10B), these compounds inhabit a binding site with a distinctive V-shape that accommodates both of the geranyl lipophilic

chains with the BP head group still positioned to interact with 2 Mg^{2+} ions. This allows considerable interaction space to be modeled for further synthetic efforts around these structures with the aim of improving selectivity and affinity [98–100]. More recently, Zhou et al. [101] have demonstrated this through the synthesis of an isoprenoid bisphosphonate ether series where the most potent compound O-citronellylgeranyl bisphosphonate (OCGBP, **39**, Fig. 10C) contained an ether based on an (*S*)-(+)-citronellyl motif replacing one of the geranyl groups of DGBP. This substitution increased the affinity for the enzyme by 2.6 fold compared to the parent (210 nM for DGBP *vs*. 82 nM for OCGBP).

2.6. Study of interactions between bisphosphonates and squalene synthase (SQS) via protein crystallography

The SQS enzyme catalyzes the condensation of two FDP (C_{15}) molecules to produce squalene (C_{30}) which is crucial for the synthesis of sterols such as cholesterol and ergosterol. Inhibitors of SQS may be of therapeutic value in the treatment of hyperlipidemia, cancer, neurode-generative disorders, and as antimicrobial agents [102]. N-BPs such as ibandronate and incadronate were reported to inhibit SQS from rat microsomes, inhibit sterol biosynthesis in mouse macrophage J774 cells, and inhibit cholesterol biosynthesis in the liver in rats [103,104]. In another study, lipophilic BPs were shown to inhibit rat microsomal SQS, inhibit cholesterol biosynthesis in rats, and lower plasma cholesterol levels in rats and hamsters [105]. More recently, the Oldfield group tested diverse compounds including *N*-alkyl imidazolium analogs of zoledronate and deshydroxyzoledronate as inhibitors of *Trypanosoma cruzi* SQS (TcSQS) for the treatment of Chagas disease [106]. The *N*-alkyl portion of the BP analogs varied from one to fifteen carbons. Zoledronate, deshydroxyzoledronate, and shorter alkyl chains analogs (C_1–C_5) were inactive against TcSQS (IC_{50} > 10 μM). The activity of the longer chain analogs increased with chain length with the C_9–C_{13} analogs showing the highest activity (IC_{50} < 0.1 μM). The corresponding zoledronate and deshydroxyzoledronate analogs had comparable activity. The most active compounds were the C_{10} zoledronate analog **41a** (BP-1237, IC_{50} = 0.018 μM) and C_{11} deshydroxyzoledronate analog **41b** (BP-1328, IC_{50} = 0.01 μM). The active analogs were generally more selective for TcSQS *vs* human SQS (HsSQS), TcFDPS, and hFDPS. For example, **41b** was 11-fold more selective for TcSQS *vs* HsSQS, 39-fold more selective *vs* TcFDPS, and 28-fold more selective *vs* hFDPS. **41b** also inhibited the growth of *T. cruzi* amastigotes (ED_{50} = 4 ± 1.3 μM) and displayed low cytotoxicity against vero cells (ED_{50} ~ 1000 μM). The authors also determined the X-ray structures of TcSQS and HsSQS complexed with representative BPs and compared these to the X-ray of the enzymes complexed with the substrate-like inhibitor **FSPP** (S-thiolo-farnesyldiphosphate, **40**). The SQS – **FSPP** X-ray shows two **FSPP** molecules in the active site – one mimicking the allylic FDP and the other mimicking the homoallylic FDP (Fig. 11). The X-ray of TcSQS – **41a** shows the compound bound to the homoallylic binding site (Fig. 12). The bisphosphonate group interacts with the side-chains of Ser42, Ser44, Tyr68, and Arg68 and the main chains of Ser44 and Phe45. The lipophilic tail extends into a hydrophobic pocket formed by Gly172, Leu175, Met199, Gly200, and Leu203 in a similar fashion as the farnesyl group of **FSPP**, and the terminal methyl of **41a** and **FSPP** end in the same position. The C_{15}-deshydroxyzoledronate analog **41c** (BPH-1344) (TcSQS IC_{50} = 0.14 μM) shows a similar binding mode as **41a** except that the alkyl chain bends at the initial carbons to

occupy portions of the allylic binding site (Fig. 12). The terminal methyl of **41c** (BPH-1344) reaches the same position as the terminal methyl of **41a** and **FSPP**. These BPs bind to HsSQS in the same manner as TcSQS. The authors identified certain differences in close proximity of the hydrophobic pocket in the allylic binding site, for example Tyr179 and Ser283 in TcSQS are substituted by Phe187 and Cys289 respectively in HsSQS, that can be exploited to develop more selective inhibitors of TcSQS.

2.7. Advances in the study of differing bone affinities among bisphosphonates and predicting structure-activity relationships: combining data from HAP mineral binding and inhibition (IC_{50}) of FDPS predicts pharmacological potency

The bone binding property of BPs is an integral part of their biological activity and it significantly influences their skeletal uptake and retention, bone distribution, and potential recycling. A variety of *in vitro* methods have been utilized over the years to study the bone affinity of BPs as reviewed in detail in our 2011 paper [2]. The results of the *in vitro* studies were significantly affected by assay conditions such as pH, buffer concentration, and methodology, which has produced some apparent inconsistencies in the rank ordering of the bone binding affinities of BPs. Nevertheless, alendronate, zoledronate, and pamidronate typically rank as high-affinity N-BPs and risedronate and minodronate as somewhat lower affinity N-BPs [107]. In an additional study of the bone binding rank order of BPs *in vivo*, Lundy et al. [108] performed a direct comparison of the skeletal binding of BPs including fluorescently labeled BPs by simultaneously administering the BPs to rats and measuring their urinary excretion using LC-MS. Approximately 30–50% of the administered dose of the highest-affinity BPs and up to 95% of the administered dose of very low-affinity BPs were found in urine within 24 h (Fig. 13). Approximately 90% of the amount excreted within 24 h was excreted within the first 4 h. The rank order for bone binding of the BPs from this analysis was: Alendronate > Etidronate ~ Zoledronate > Neridronate > Risedronate > Minodronate > FAM-RIS ~ Clodronate > Ibandronate > OX14 ≫ NE-10790. Generally there was an inverse relationship between urinary excretion and affinity to HAP, indicating that binding of BPs to HAP strongly influences their *in vivo* distribution and excretion profiles [28,108]. While there are some discrepancies among typically described bone affinity measurement methods (*e.g.*, for etidronate, ibandronate), it is still not known how much protein binding might be a confounder as well as the balance between kinetic binding *vs* thermodynamic retention in this *in vivo* analysis [5].

As discussed earlier in this review, the two key properties of BPs: FDPS inhibitory activity and bone binding affinity, play the major role in determining the overall antiresorptive efficacy of BPs *in vivo*. Thus, the predictive potential of the *in vivo* efficacy of a novel BP molecule from *in vitro* assays has been addressed [108]. Similar to previous modelling, that was based on the combination of the IC_{50} of FDPS inhibition and the measure of bone affinity obtained from the HAP affinity column as discussed in our 2011 review [2,111], the relationship between predicted D_{20} and experimental D_{20} (D_{20} is the dose calculated from a dose-response study necessary to change BMD (assessed using DEXA) by 20% *vs* control animals in the growing rat model) was reanalyzed by Mark Lundy and Lin Fei using a multiple regression model with additional bisphosphonate analogs. By using the binding affinity data obtained from the HAP affinity column assay and FDPS

inhibition data (IC_{50}) to calculate the predicted D_{20}, the striking correlation to the *in vivo* efficacy (experimental D_{20}) was reinforced ($r^2 = 0.90$) (Fig. 14) [108]. This result further demonstrates the prediction utility of such correlation modelling and that these two parameters (FDPS inhibition and bone affinity) are the major drivers of the pharmacological potency of bisphosphonates *in vivo*. This better understanding may therefore enable more efficient screening of new analogs for their desired *in vivo* efficacy profile.

3. New directions in bisphosphonate research

3.1. New uses of bisphosphonates and their relation to FDPS or other mechanisms

Now with many years of patient experience with bisphosphonates, and multiple prospective and epidemiologic clinical analyses, along with the advanced understanding of the mechanism of action of the bisphosphonates [3], opportunities for their use in new directions are receiving increased attention, both in skeletal diseases and even in non-skeletal fields. This includes immunomodulatory, anti-viral, and anti-tumor activities, and effects on protozoan parasites. For example, further studies have recently shown that N-BPs suppress the spread of various cancer cells *in vitro* [50,51,112,113]. However, it is not clear that the relatively high concentrations used in these studies are attainable *in vivo*. Others have discussed their ability to activate certain T-cell which may be related to an “acute phase response” sometimes observed on initial clinical dosing with iv bisphosphonates [114,115].

Promising nonclinical results with newly synthesized α-amino bisphosphonates raise hopes for the development of novel bisphosphonates with useful antioxidant, antimicrobial, and anti-cancer properties. Sudileti et al. have studied the anticancer effects of series of fluoro-substituted α-amino bisphosphonates against the human breast cancer cell lines (MCF-7) using the MTT assay method [51]. They found compounds **42**(**a**–**f**) (Fig. 15) have shown greater antiproliferative effects *in vitro* as compared to the anticancer drug exemestane. In a different study, Lacbay et al. [116] have presented α-amino bisphosphonate **37a** (Fig. 15) as a new class of inhibitors of hGGPPS and have reported the antimyeloma effects of **37a** both *in vitro* and in multiple myeloma (MM) disease mouse models, as discussed in the section of “BP-GGPPS interaction” above. The study of antimicrobial activity of a series of α-amino bisphosphonates by Tellamekala et al. [49] have shown that compounds **43c**, **44**(**a**–**b**), and **45** displayed superior anti-fungal activity to that of voriconazole. Similarly, compounds **43**(**a**–**d**) exhibited excellent antioxidant activity compared to that of ascorbic acid (Fig. 15). Moreover, recent computation docking studies conducted by Shaik et al. have predicted that compounds **46**(**a**–**b**) (Fig. 15) have stronger inhibitory activity on human DNA ligase enzyme than that of the antibiotic ciprofloxacin [48].

In the past decade, a series of lipophilic bisphosphonates was synthesized, among which some of the triazole-based ones have shown exceptional inhibitory activities towards enzymes in the mevalonate pathway (Fig. 16). Assembly of triazole-based bisphosphonates with lipophilic tails can be obtained through a click chemistry reaction between an azide and an acetylene. In a report by Zhou et al. [117], compound **47** has shown potency towards inhibiting GGTase II with an IC_{50} of 0.1 mM, while **48** is a potent and specific inhibitor of GGPPS with an IC_{50} = 0.38 μM [118]. In 2015, Wills and coworkers reported that compound **36** (a 3:1 mixture of *E* and *Z* olefin isomers) shows an excellent inhibitory

effect against GGPPS with an IC_{50} = 45 nM, which thus far is the most potent GGPPS inhibitor reported [96]. Later, in 2018 Matthiesen et al. modified the structure of **36** by incorporating a methyl group at the α-carbon to obtain **49a** (*Z* isomer) and **50a** (*E* isomer) [119]. This modification has slightly reduced the GGPPS inhibitory of **49a** (IC_{50} = 85 nM) and **50a** (IC_{50} = 125 nM); however, α-methylation allowed the preparation of POM-prodrugs (**49b** and **50b**), which exhibited a 10-fold increase in cellular activity compared to the corresponding salt.

Other observations suggesting potential nonskeletal effects with the current clinically used bisphosphonates have also appeared in the literature in recent years. An exciting recent report discusses the evidence for the mesenchymal stem cell protective effects of the bisphosphonates and their ability to block DNA damage and enhance stem cell repair after irradiation or other damage [120]. Inhibition of FDPS within these cells appears to be an important step leading to these effects. Examples of enhanced wound healing and perhaps even anti-fibrotic activity have been demonstrated in models of ulcerative colitis [121], lung fibrosis [122], and lifespan and cardiac benefits [123]. The finding that inhibition of GGPPS is a potential treatment for pulmonary fibrosis has recently been followed with a report showing that simply increasing the flux of metabolites through the pathway of isoprenoid synthesis leads to increased macrophage-mediated fibrosis [124]. This result points to the use of GGPPS inhibitors in fibrotic diseases in general where this type of macrophage dysregulation mediates the pathology. Thus, advances in the chemistry of bisphosphonates and our advanced mechanistic understanding of enzyme targets may yield future designer bisphosphonates in these fields, perhaps with lower bone affinity and higher targeting to other tissue compartments.

Furthermore, there are also many new opportunities for improved studies and new data for bisphosphonates in the veterinary field, as reports of their use in pets, horses, and other domestic animals are increasing [125,126]. Surprisingly little work has been done to develop bisphosphonate drugs for uses in this field. Additional work is clearly needed for generating optimized veterinary products.

3.2. Fluorescent BP analogs and applications

Over the past two decades, many more fluorescent bisphosphonates have been designed and made available as probes for mechanistic studies and as diagnostic research tools [2]. The McKenna lab introduced a facile “magic linker” strategy to create the first fluorescent conjugates of a modern N-BP drug, RIS bisphosphonate as imaging tools [110]. Since then, additional fluorescent bisphosphonates were prepared, expanding the “toolkit” to cover all clinically used N-heterocyclic BPs (ZOL, MIN) and related analogues, as well as a wider spectroscopic window from visible to near-infrared range (Fig. 17) [109]. These probes have been used in various imaging studies in skeletal-related fields [127–130], and further expanded in non-skeletal fields in recent years, including dental research [131–136], otology [137–139], drug distribution studies [140,141], cancer research [142,143], nephrology [144] and brain calcification studies [145].

Another noteworthy highlight concerning the use of fluorescent bisphosphonates is the contribution they offer to a more in-depth understanding of how structure-activity

relationships among bisphosphonates influence their skeletal distribution. This may lead to new avenues of therapeutic exploration, for example in gaining access to osteocytes within the canalicular network in bone [128,129]. Of course fluorescent BPs may not only have diagnostic potential but also combined therapeutic/diagnostic value as well. For example, McKenna and collaborators have exploited the fact that one bisphosphonate can displace another on the bone surface, which has led to the development of an “antidote” procedure [131]. By utilizing relatively inactive (low potency) bisphosphonates (*e.g.*, some fluorescent bisphosphonates) that maintain their bone binding affinity, it has been shown that this strategy may aid the prevention and treatment of related symptoms of zoledronate associated osteonecrosis of the jaw in a rodent model [136]. In addition, a recent significant study by McDonald, et al. reported a novel cellular program for osteoclast reuse through their fission, transport, and reassembly process (osteoclast-osteomorph recycling) [146]. This finding redefines the understanding of osteoclast physiology and also points to new actionable targets for novel therapeutic development [147]. Fluorescent bisphosphonates have played an indispensable role in this project as imaging probes, which enable direct observation of the process *via* intravital imaging technique.

These fluorescent bisphosphonates are “always on” in the sense that their fluorescence persists wherever they may bind. The complementary concept of an imaging bisphosphonate whose fluorescence is “off” until triggered by a specific enzyme targeted *in situ* was recently demonstrated by McKenna and collaborators, who synthesized a molecule consisting of a Förster internally-quenched dye pair linked by a cathepsin K peptide substrate attached to a bisphosphonate, to detect *in situ* bone resorption by osteoclasts, which secrete proteolytic cathepsin K to break down the collagen matrix in the bone. This “osteoadsorptive fluorogenic sentinel” or OFS probe adsorbed on hydroxyapatite generated a localized fluorescent signal when activated by a resorbing osteoclast. Its exciting diagnostic potential was shown by its ability to sensitively image multiple myeloma cells metastasized to a nascent bone tumor site in a murine model [148].

3.3. Targeting drugs to the bone with bisphosphonates

Ideally, drugs should act directly upon their intended targets, selectively in the relevant tissues, in order to achieve optimal efficacy with minimal side-effects. There are several ways to pursue this in drug design, but this goal is not always straightforward. With statins, for example, selective liver uptake is known to be a desirable property, but off-target effects on muscle can lead to side effects. Key principles underlying the development of selective bone-targeted bisphosphonate – drug conjugates continue to evolve [6,149], and more recent successes with *in vivo* “Target and Release” strategies offer an exciting tissue-selective drug design approach [150].

The risk of off-target toxicities with non-selective drug classes can also hamper the drug discovery process, particularly for early *in vivo* evaluation of a drug class. In the case of drugs acting on or within the bone, the bisphosphonates offer an obvious and unique opportunity for selective drug delivery. In theory, selectivity from targeting bone with bisphosphonates could be achieved through their conjugation with many different relevant drug classes. In practice, the resulting bone-targeted drugs may offer important new

therapies for many unmet medical needs. While this concept is not new, there are significant challenges with this drug design approach and it has achieved only limited clinical success. The permanent covalent linking of two drug classes generally can significantly diminish the activity of each component in drug conjugates. Efficient and effective release mechanisms are likely to be needed in most cases as precise local concentrations of released drug need to be generated which complicates the pharmacokinetics of releasable BP-drug conjugates. These hurdles have led to a slower than desired evolution of such a bone targeting strategy in drug design [151].

Only recently, advances in chemistry have enabled the identification of bisphosphonate linkers that provide adequate stability in the bloodstream, and rapid enough break-down and release of pharmacologically active agents at the bone surface in relevant concentrations to affect the desired biochemical targets. These new conjugates are being designed to release active drugs in the vicinity of cells near the bone surface in a “depot” fashion. The repertoire of bisphosphonate conjugates of various known and experimental drug classes includes steroids [152], prostaglandins [153], antibiotics [154,155], and several anticancer drug classes [156]. Such an approach may also enhance the likelihood of success in new drug design by offering more rapid *in vivo* validation of new drug classes and their targets for further optimization of drug leads. Therefore, it is likely that further research on the development of bone-targeted drugs for a variety of bone-related diseases will offer potential new therapeutic opportunities. In particular, potential new treatments of multiple myeloma and other bone metastatic cancers, osteomyelitis, arthritis, fracture repair, and osteoporosis are currently being pursued. Several recent examples have built upon historic attempts of drug development with this strategy, and exemplify many of these principles with new “proof of concept” *in vivo* data. In particular, linkages between the bisphosphonate and the active drug have been optimized for adequate stability in the circulation, but with useful lability at the bone surface to yield effective local concentrations of the drug. Furthermore, pharmacologically less active bisphosphonates have been utilized recently, in order to minimize confounding activities, concerns about differing relative dose levels for each component, and to avoid any un-toward effects of the bisphosphonate. Thus, the bisphosphonate is primarily used as a carrier in some of the recent examples of *in vivo* “proof of concept” studies described below.

The development of a bone-targeted drug for use in multiple myeloma (MM) would represent a potential medical breakthrough for the treatment and management of MM, and also, perhaps, for other haematopoietic malignancies or primary bone tumors. First, the future drug would augment existing drug treatments by addressing simultaneously the detrimental hallmarks of these tumor types, namely infiltration of bone tissue by cancer cells and induction of excessive bone resorption, which in turn leads to increased skeletal complications, including bone destruction, fractures, and pain. Second, a tissue-targeted strategy will lower systemic drug levels, resulting in reduced adverse side effects, an important factor that in clinical practice often limits efficient treatments. Third, the ability to selectively and directly modulate and reshape the tumor microenvironment (including bone tissue, osteoclasts, osteoblasts, and immune cells), addresses the challenges in MM therapy, where relapse and development of therapy resistance are thought to be critically dependent on the complex changes occurring in the cancer microenvironment.

A team at Rochester recently pursued a bone targeting approach for the treatment of multiple myeloma with the design of a bisphosphonate linked bortezomib analog *via* a linker that appears to have significant bloodstream stability, but which steadily releases bortezomib at the bone surface due to acid instability of the boronate ester linkage (Fig. 18, **BP-Btz**, compound **52**) [157]. Notably, a dramatic reduction in soft tissue toxicities were observed relative to equivalent doses of bortezomib. This approach has also been evaluated as a new bone formation stimulant with exciting results in animal models [158].

The Rochester team has also optimized a carbamate linkage to a low activity bisphosphonate to chloroquine (Fig. 18, **BP-CQ**, compound **53**) and hydroxychloroquine (Fig. 18, **BP-HCQ**, compound **54**) [150,159]. *In vitro*, these conjugates prevented osteoclast formation whereas a stable amide-linked conjugate was nearly inactive in this respect. Enhanced *in vivo* efficacy was also observed with the conjugates when compared to chloroquine/hydroxchloroquine.

Collaborating BioVinc/USC research teams recently designed, synthesized, and evaluated a novel bone-targeting antimicrobial agent comprising a pharmacologically “inert” bisphosphonate (BP) moiety that was conjugated – through metabolically hydrolyzable aryl carbamate linkers – to a fluoroquinolone (FQ) antibiotic, ciprofloxacin, *via* another “target and release” chemical strategy (Fig. 18, **BV600022**, compound **55**) [160]. These linkers are relatively stable in the bloodstream, while the antibiotic is delivered to metabolically active bone surfaces, but are cleaved presumably by the enzymatic environment produced by both the bone-resorbing cell, the osteoclast, and at the bacterial cell bone surface interface. FQ antibiotics remain a mainstay for the treatment of bone infections in adults, particularly first-line oral monotherapy with agents like ciprofloxacin, levofloxacin, and moxifloxacin. As noted above, a pharmacologically inactive BP was used as a bone targeting carrier for this purpose. Also, it was further demonstrated that BPs are particularly ideal for targeting anti-infective agents to the bone surfaces where biofilm infections occur, not only because they have a high affinity for hydroxyapatite (HA) and selectively bind to bone, but also because they preferentially accumulate at sites of active bone disease or infection, where the highest turnover is taking place [161].

BPs also penetrate the canalicular network to osteocytes and osteocytic lacunae where no blood flow exists and where *S. aureus* organisms, in particular, are known to embed [162,163]. Accordingly, the demonstrated efficacy of using a novel bone-targeted BP-FQ conjugate for the treatment of osteomyelitis biofilms is a particularly good demonstration of a new therapeutic opportunity for such “custom designed” bisphosphonates. Thus, *in vitro* antimicrobial susceptibility testing revealed an effective bactericidal profile and sustained release of the parent antibiotic over time. Efficacy and safety were demonstrated in an animal model of jawbone periprosthetic osteomyelitis, where a single dose of 10 mg/kg (15.6 μmol/kg) conjugate reduced the bacterial load by 99% and demonstrated nearly an order of magnitude greater activity than the parent antibiotic alone given in multiple doses (total dose: 30 mg/kg, 90.6 μmol/kg) [160].

Another academic research team has recently reported studies of a bisphosphonate-targeted gamma-secretase inhibitor analog GSI-XII (Fig. 18, **BT-GSI**, compound **56**). This “target

and release" approach was designed to release this Notch inhibitor near osteoclasts where the acid sensitive linker cleaves at the bone surface, and it has been shown to bring enhanced *in vivo* activity to this ubiquitous and relatively toxic series of Notch inhibitors. *In vivo* efficacy was demonstrated in a mouse model of multiple myeloma and no gastrointestinal (GI) side effects typically associated with GSI-XII alone were observed [164,165].

4. Conclusions

Studies of the structure-activity relationships of bisphosphonates over many years have led to a better understanding of their unique properties and how they work. Continued efforts in synthetic chemistry have provided additional methods for more efficient or "greener" approaches to produce new BPs for scientific and potential clinical use. It has also enlarged the structural repertoire of BP molecules, enabling further research and understanding of their mechanism of action, as well as expanding their uses in more fields. The identification of the major enzymatic target, FDPS, for the nitrogen-containing BPs, was probably the most remarkable landmark achievement along the way. Further efforts to elucidate the interactions between BPs and FDPS, and other important enzymes in the mevalonate pathway (GGPPS, SQS) *via* crystallography, and new HAP crystal physical chemical interactions have augmented our knowledge of the structure-activity relationships, and also indicated potential new uses of BPs in other disease areas.

With the advanced understanding of these mechanisms of action, we now have a clearer consensus about the two key properties responsible for their antiresorptive efficacy (bone affinity and cellular effects). We can now more clearly explain their clinical features, and can rationally design novel BPs with desired pharmacological properties. New therapeutic areas that could potentially benefit from this optimized and rational drug design include bone-related and other cancers, inflammatory and arthritic conditions, viral infections, and the intriguing possibility of altering age related health span, by extending longevity and reducing multimorbidity.

Another area of growing importance and research attention is the ability of conjugated bisphosphonates to act as carriers to target other drug classes to the bone, due to their exceptional selectivity for bone as the target organ. Although this concept is not new, additional clinical success remains to be accomplished with this approach. A classical application that has long enjoyed clinical success is the technetium-BP bone scanning using SPECT. Recent advances in conjugating BPs with fluorescent dyes have proven to be invaluable research tools for elucidating BP drug pharmacology and hold promise for eventual development as clinical imaging probes. It is encouraging that significant work has been done in the last decade and is receiving increasing interest. A recent emphasis on conjugates that provide a "target and release" of active drugs at bone surfaces is offering unique and exciting treatment possibilities for several medical problems where current therapies could be significantly improved, such as bone infections and bone metastatic disease. Success with these endeavours would open a new era of broadening applications of new medicines from the extensive range of molecular structures based on BP molecules.

As is evident from this review, chemistry continues to play a backbone role for BP related research by providing important molecular design and new pharmacological leads. The integrated expertise of biochemical, preclinical, clinical, and pharmacology researchers in the musculoskeletal and related fields should enable a bright future for this unique class of compounds.

In conclusion, we anticipate that the fascinating scientific journey that began 50 years ago will continue well into the future.

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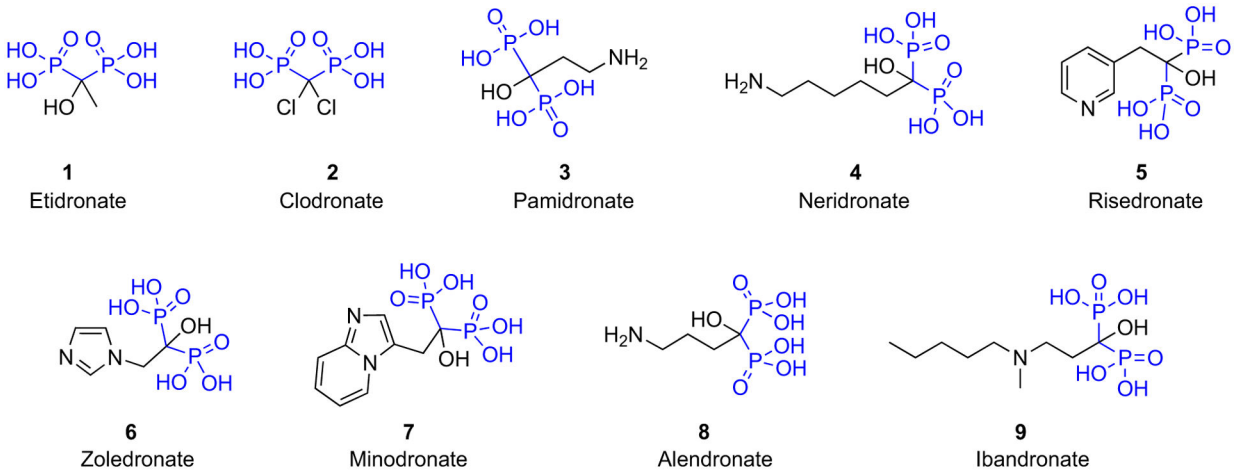


Fig. 1.
Clinically used bisphosphonates.

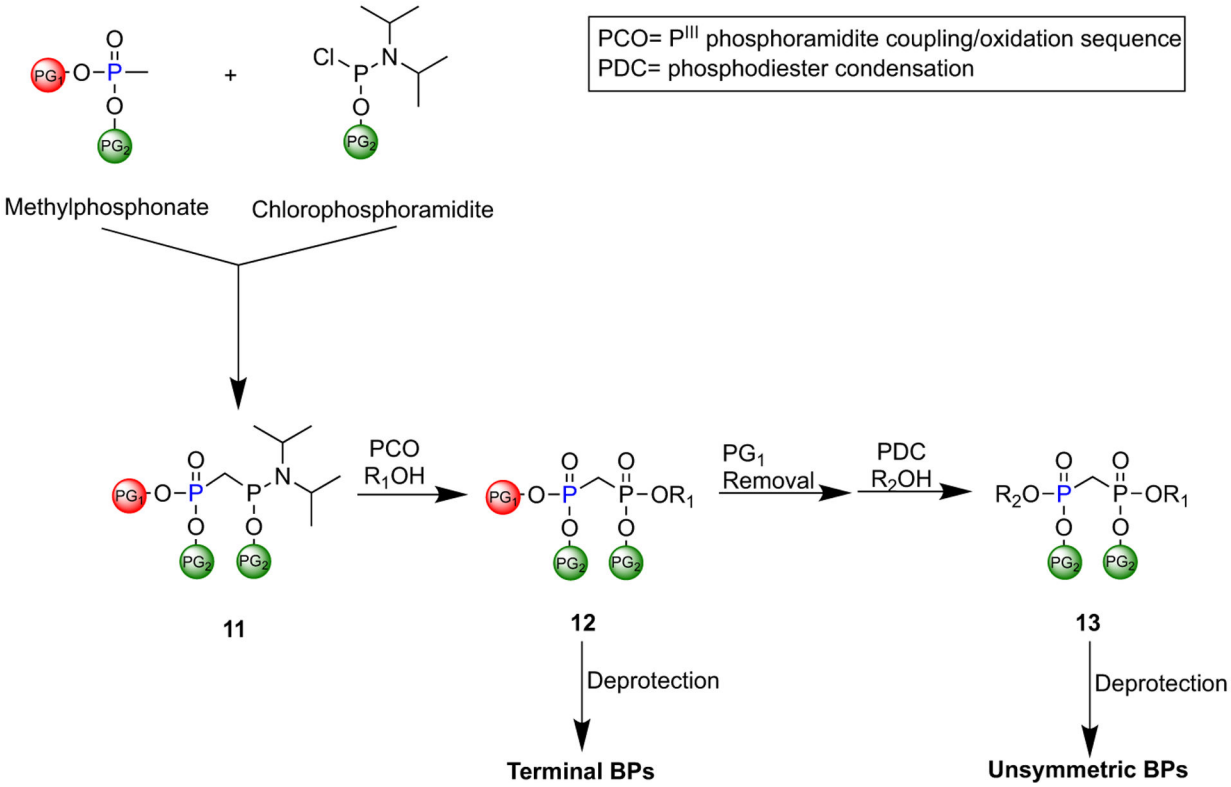


Fig. 2.

A novel protocol reported by Engelsma et al. for the synthesis of terminal and unsymmetric BPs.

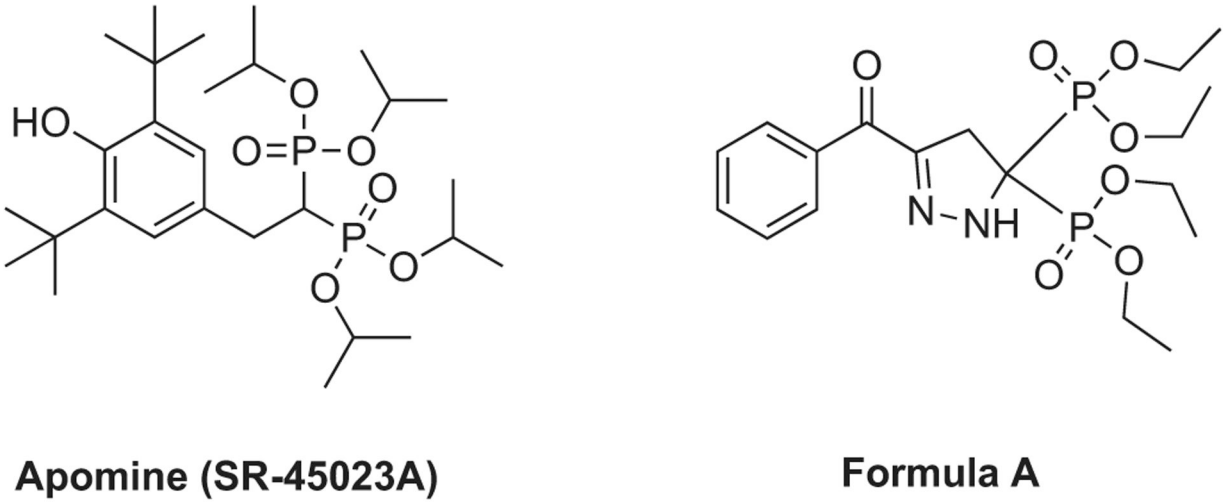


Fig. 3.

Examples of bisphosphonate esters: apomine (SR-45023A) and pyrazoline derived bisphosphonate ester (Formula A).

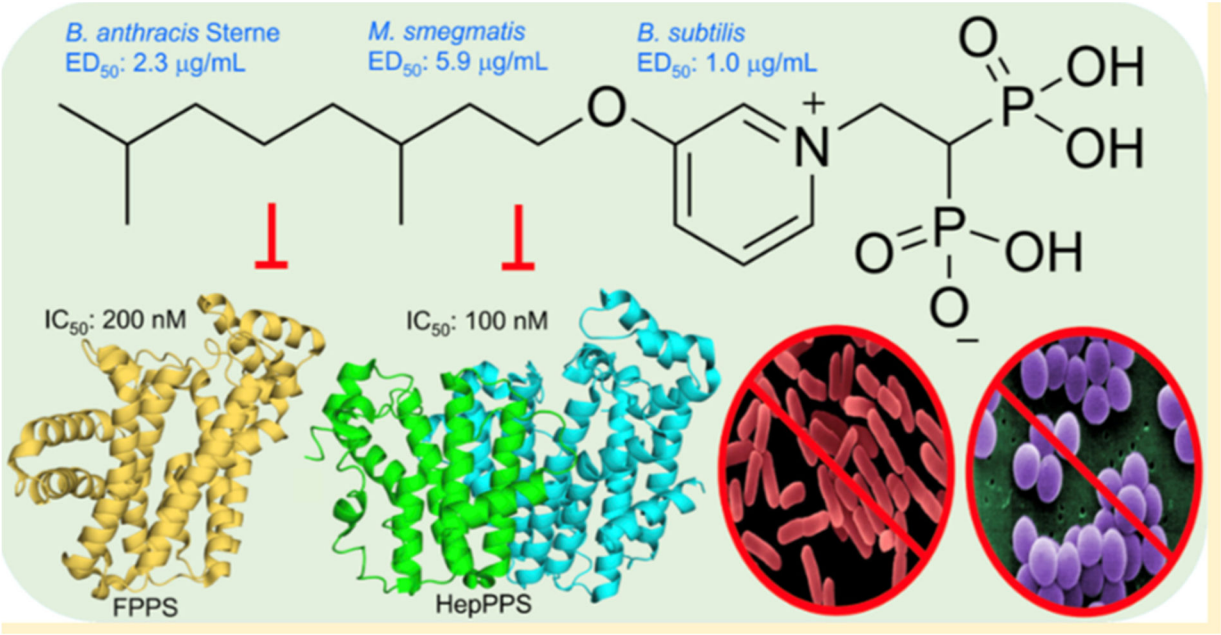


Fig. 4.

Lipophilic antibacterial bisphosphonates that target the cell wall. (Adapted from ref. [86]).

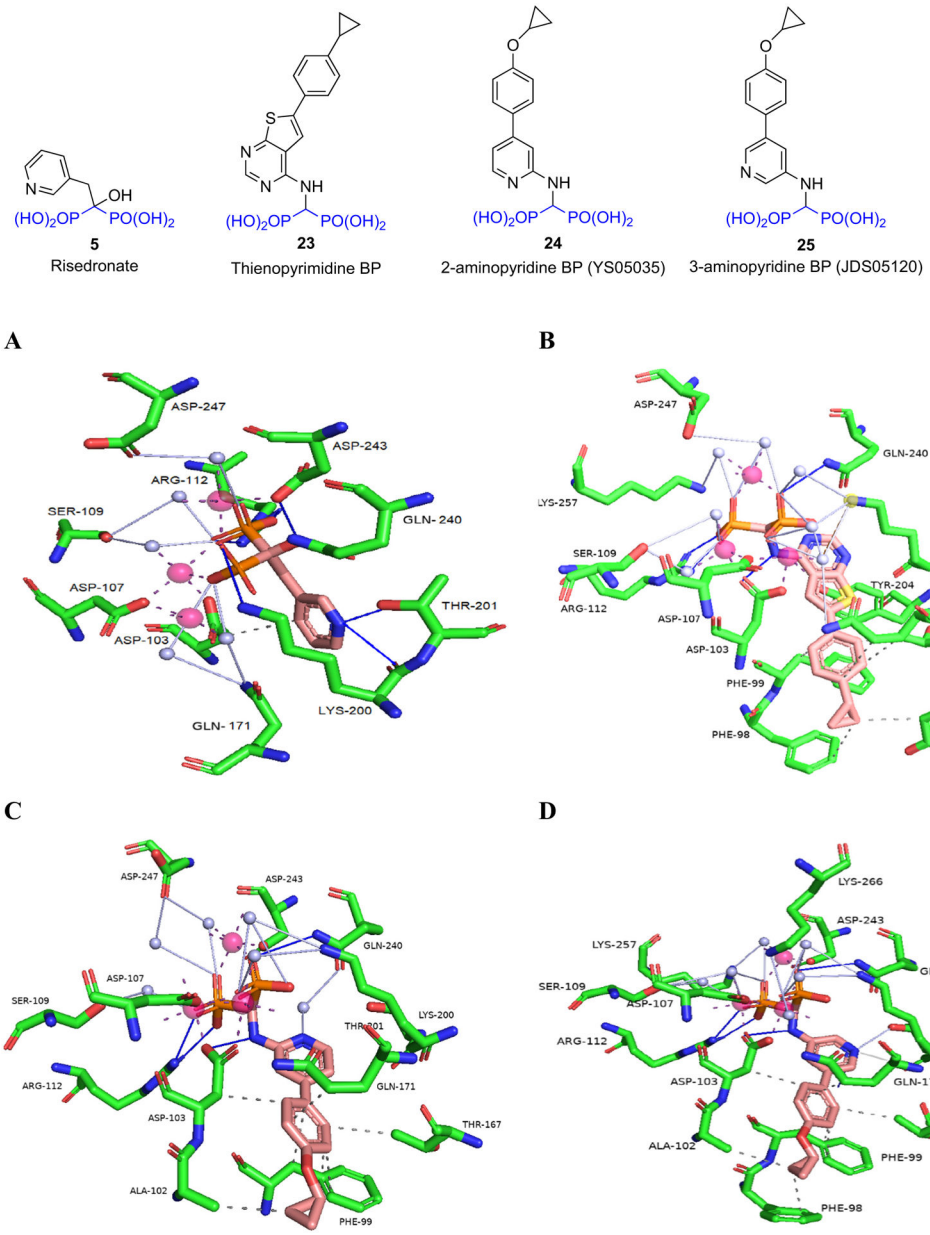


Fig. 5.

Binding of BPs to FDPS: A) Risedronate (**5**) (PDB ID 1YV5); B) Thienopyrimidine BP (**23**) (PDB ID 4L2X); C) 2-aminopyridine BP (**24**) (PDB ID 4PVX); D) 3-aminopyridine BP (**25**) (PDB ID 4NFI). Figure was generated using Protein-Ligand Interaction Profiler (PLIP) and Pymol. Code: blue line – hydrogen bond; grey line – water bridge; dashed line – hydrophobic interaction, white sphere – water molecule, pink sphere – Mg^{2+}.

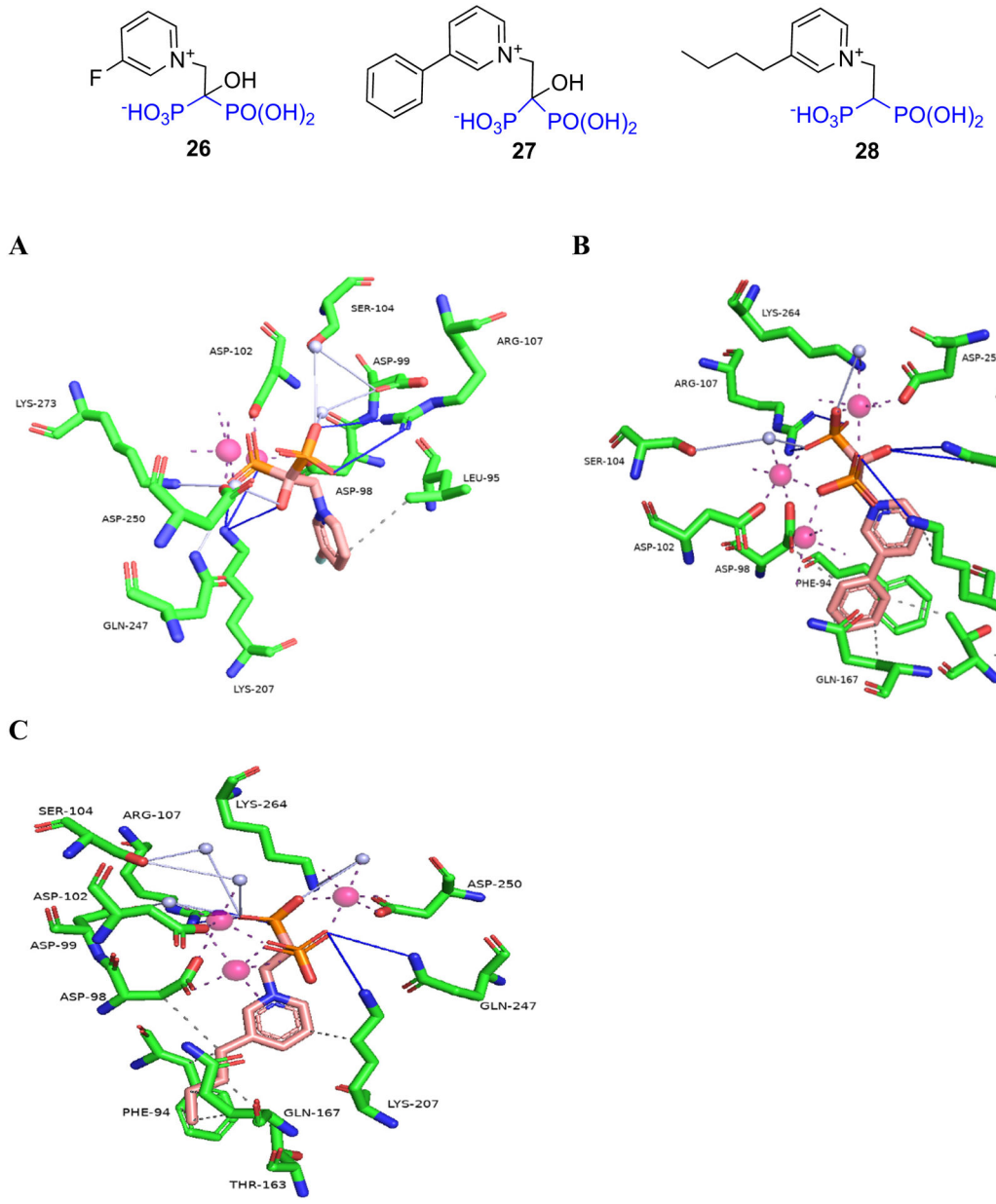


Fig. 6.

Binding of pyridinium BPs to *Leishmania major* FDPS: A) **26** (PDB ID 4K10); B) **27** (PDB ID 4JZB); C) **28** (PDB ID 4JZX).

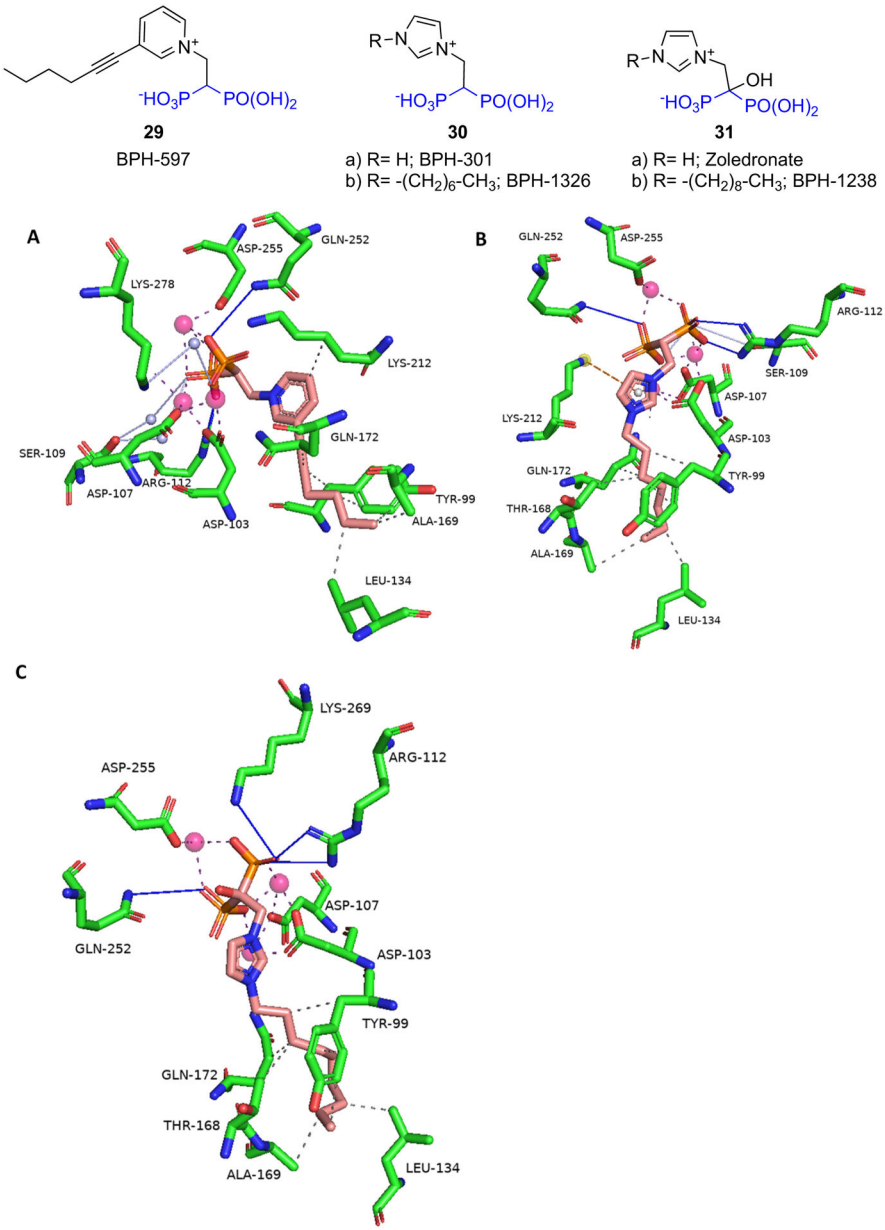


Fig. 7.

Binding of BPs to *Trypanosoma brucei* FDPS: A) **29** (BPH-597) (PDB ID 5AEL); B) **30b** (BPH-1326) (PDB ID 5AHU); C) **31b** (BPH-1238) (PDB ID 5AFX).

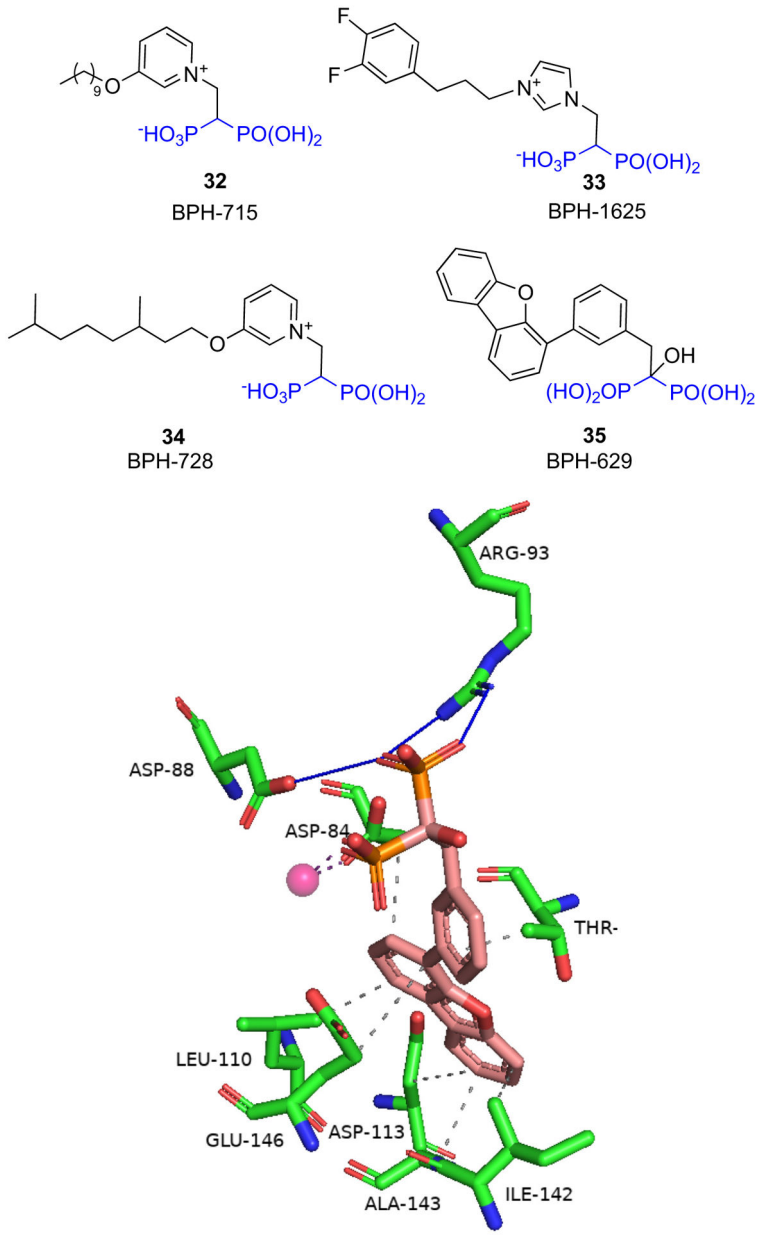


Fig. 8.
Binding of **35** to *E. coli* OPPS (PDB ID 5ZLF).

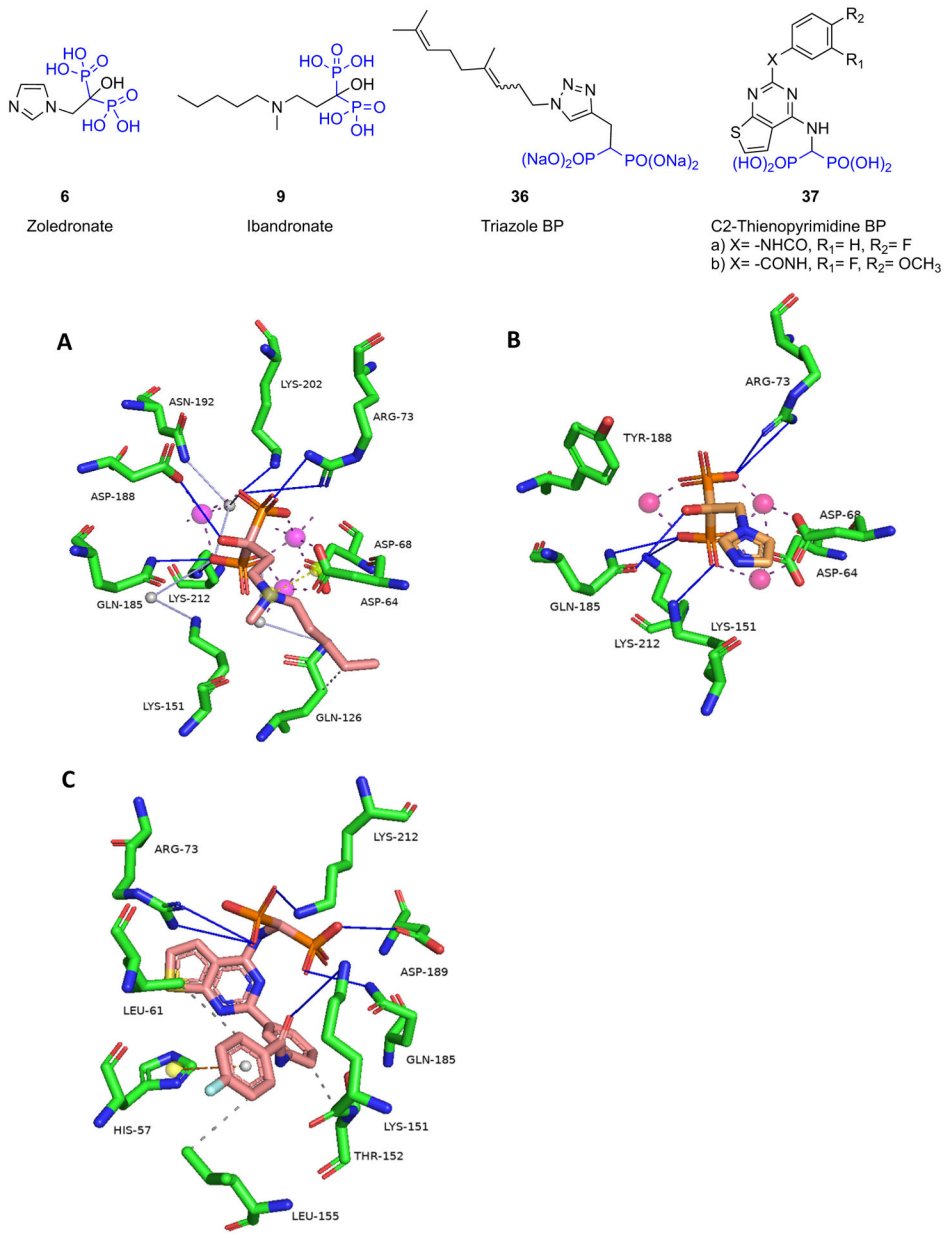


Fig. 9.

A) X-ray of hGGPPS – ibandronate (**9**) (PDB ID 6R4V); B) X-ray of hGGPPS (D118Y) – zoledronate (**6**) (6G31); C) X-ray of hGGPPS (Y246D) – **37a**. Note: yellow sphere represents a charge center.

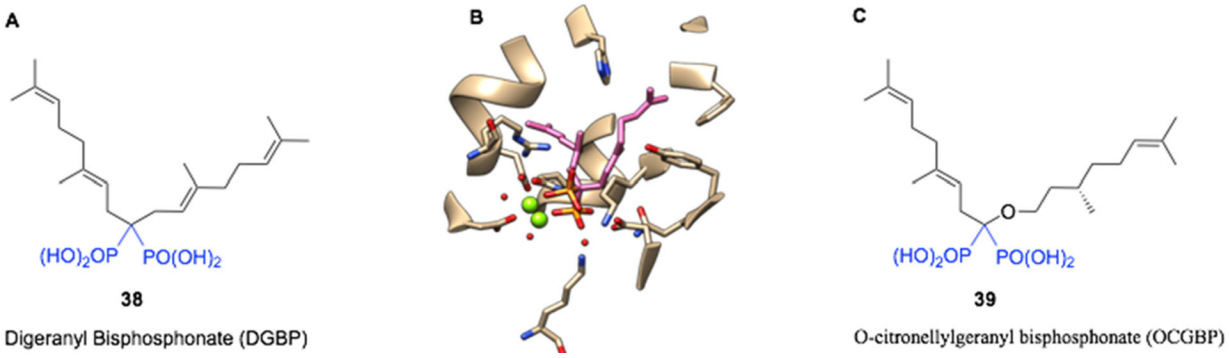


Fig. 10.

A: Structure of Digeranyl bisphosphonate (**DGBP**), B: Crystal structure of DGBP bound to *S. cerevisiae* GGPPS (pdb file 2z4w), and C: The more potent O-citronellylgeranyl bisphosphonate (**OCGBP**).

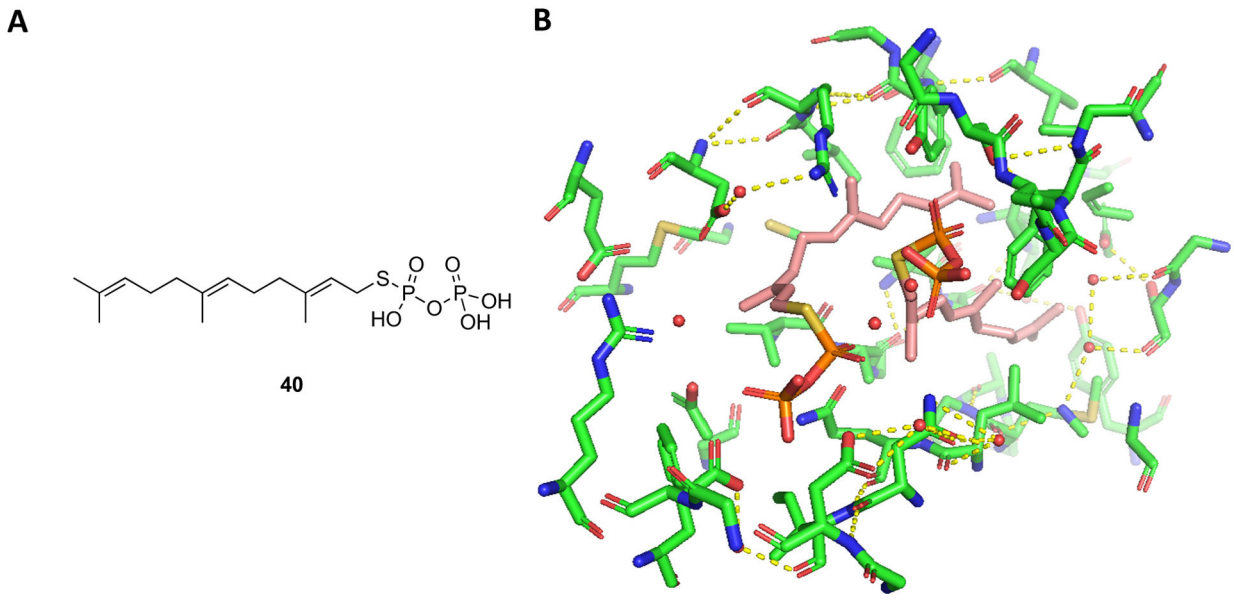
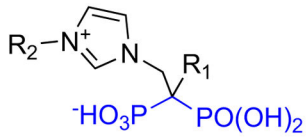


Fig. 11.

A) Structure of FSPP (**40**); B) Two molecules of FSPP bound to the active site of TcSQS (PDB ID 3WCA).



41

a) R_1= OH; R_2= -$(CH_2)_9$-CH_3; BPH-1237
b) R_1= H; R_2= -$(CH_2)_9$-CH_3; BPH-1328
c) R_1= H; R_2= -$(CH_2)_{14}$-CH_3; BPH-1344

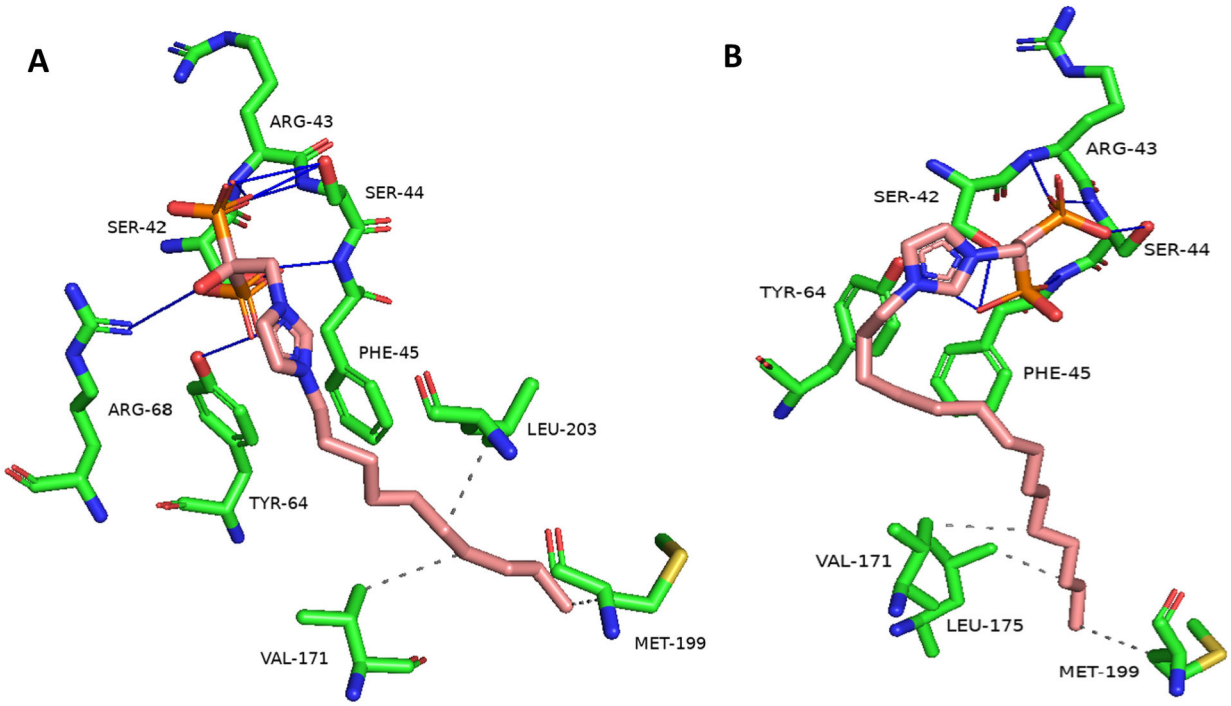


Fig. 12.

A) X-ray of TcSQS – **41a** (BPH-1237) (PDB ID 3WCB); B) X-ray of TcSQS – **41c** (BPH-1344) (3WCG).

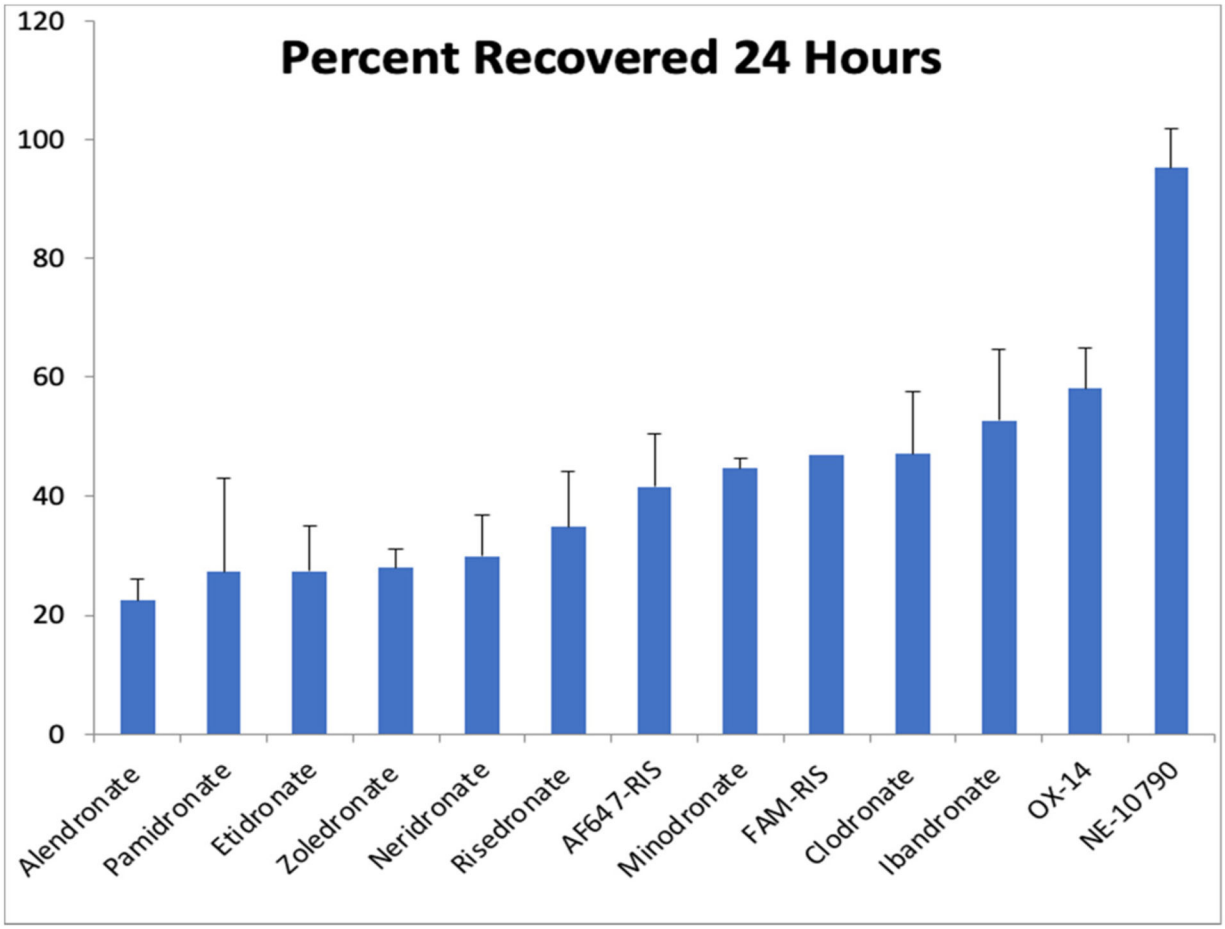


Fig. 13.

Percentage urinary excretion of bisphosphonates collected 24 h after i.v. injection (multiple compounds were administered in the same injection) (adapted from ref. [108]). Higher affinity compounds have a lower recovery in the urine. AF647-RIS (compound **52a6** in Fig. 17) & FAM-RIS (compound **52a1** in Fig. 17) are fluorescently labeled bisphosphonates [109,110].

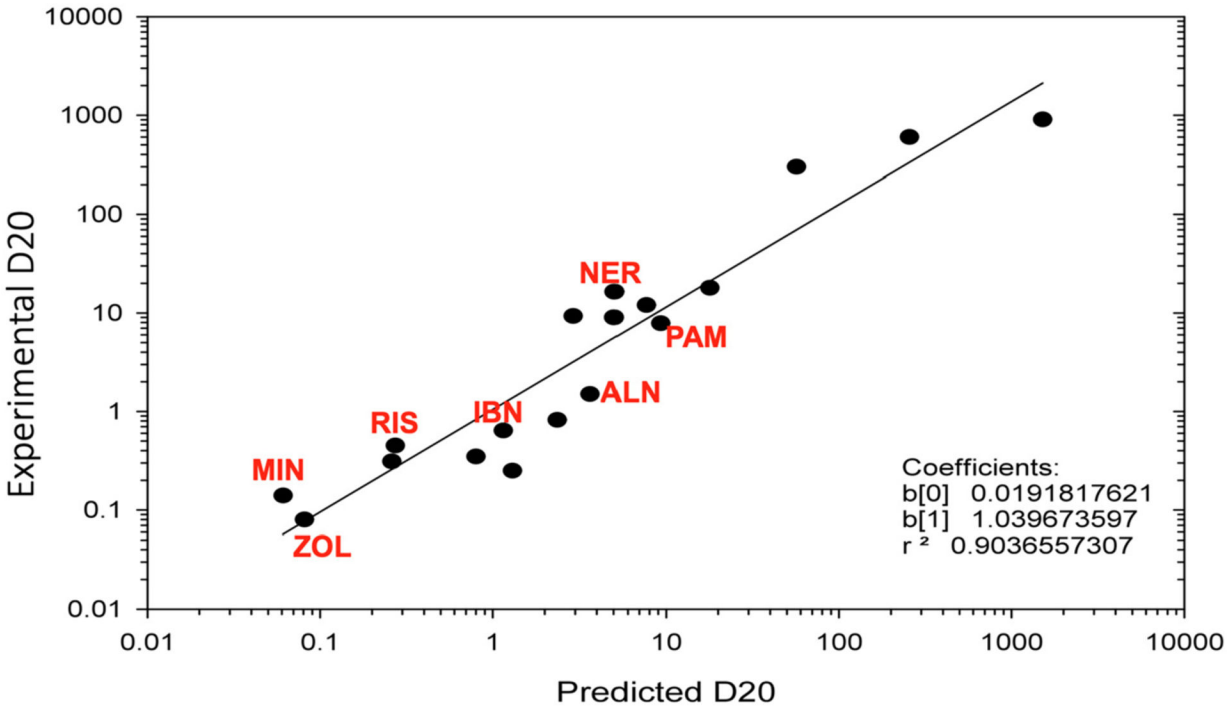


Fig. 14.

Correlation model of experimental D_{20} and predicted D_{20} (predicted D_{20} (pD_{20}) is calculated from both *in vitro* FDPS inhibition and bone affinity data, *via* formula $\log(pD_{20}) = -0.801 + 1.2605 * \log(\text{FDPS IC50 (nM)}) - 0.0656 * \text{Affinity} - 0.01841 * \log(\text{FDPS IC50 (nM)}) * \text{Affinity})$. This model allows a very accurate estimate of the anti-resorptive *in vivo* efficacy (experimental D_{20}), enabling rapid screening of new analogs based on *in vitro* data. Each dot represents a bisphosphonate analog, and all clinically used bisphosphonates are annotated in red: PAM – pamidronate (**3**), NER – neridronate (**4**), RIS – risedronate (**5**), ZOL – zoledronate (**6**), MIN – minodronate (**7**), ALN – alendronate (**8**), IBN – ibandronate (**9**).

(Figure adapted from ref. [108]).

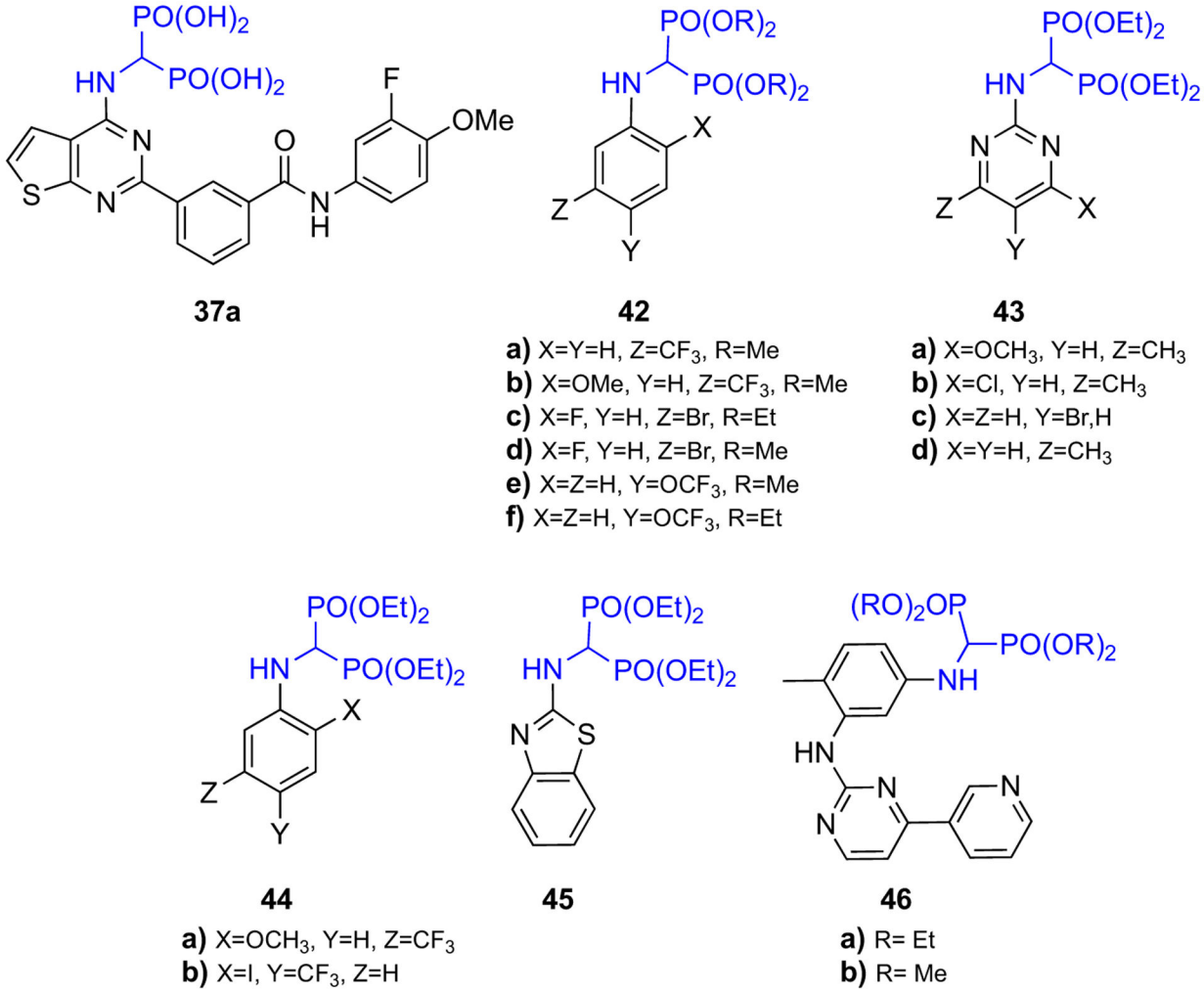


Fig. 15.
Structures of bioactive α-amino bisphosphonates.

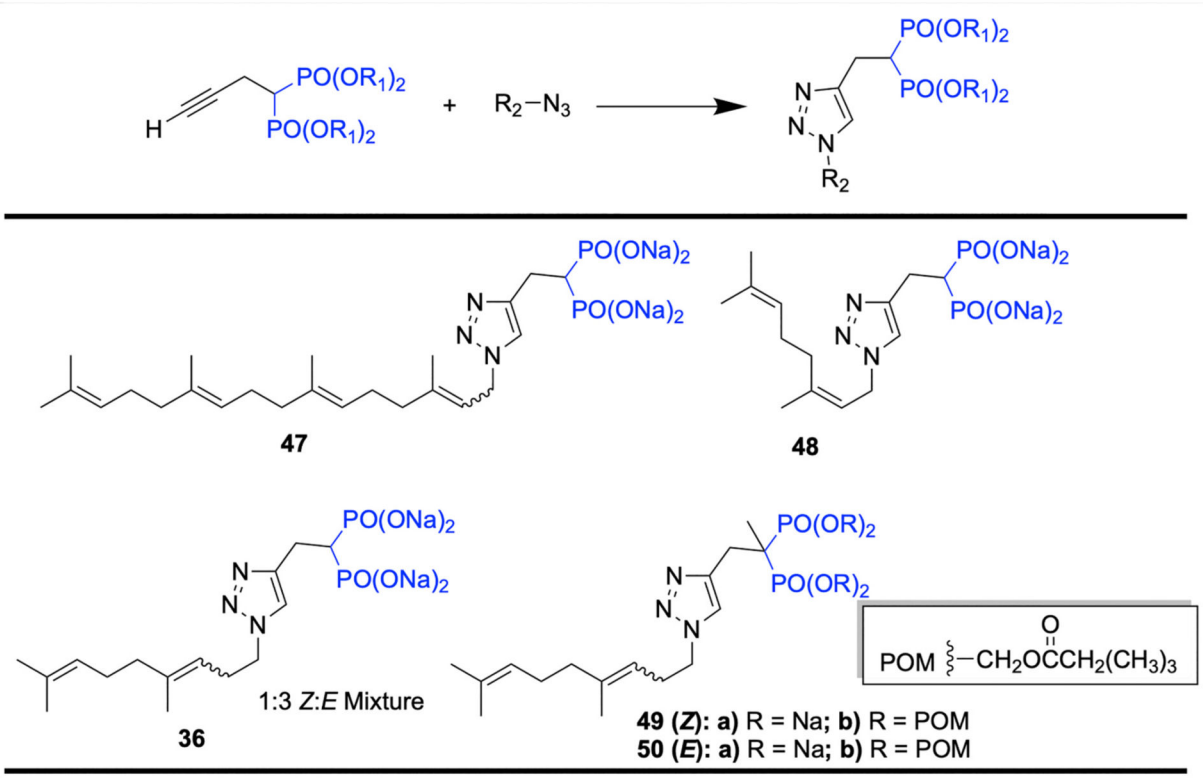
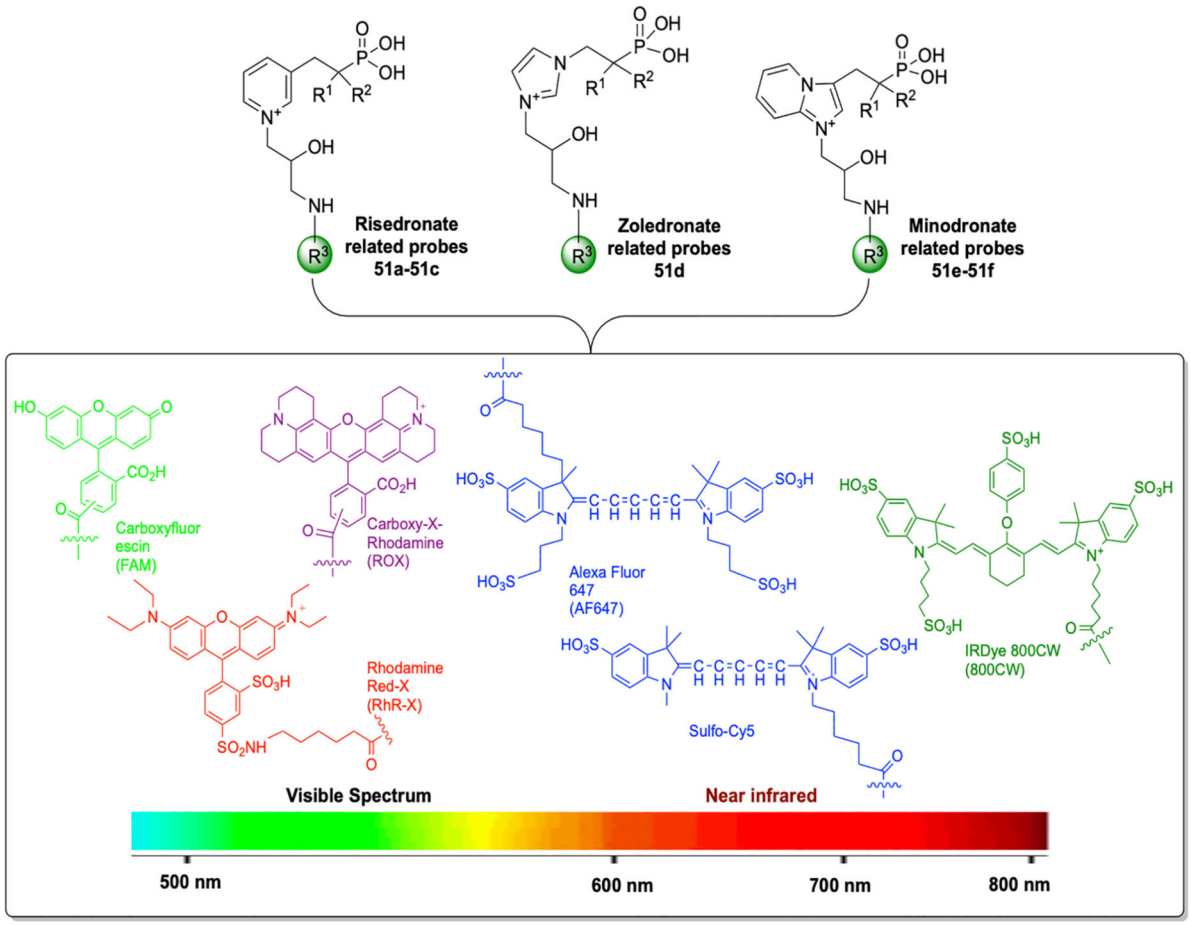


Fig. 16.
Triazole based lipophilic bisphosphonates.



51a1: 5(6)-FAM-RIS. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = FAM
51a2: 5-FAM-RIS. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 5-FAM
51a3: 6-FAM-RIS. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 6-FAM
51a4: 5(6)-RhR-RIS. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = RhR-X
51a5: 5(6)-ROX-RIS. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = ROX
51a6: AF647-RIS. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = AF647
51b1: 5(6)-FAM-RISPC. R^1 = OH, R^2 = CO_2H, R^3 = FAM
51b2: 5(6)-RhR-RISPC. R^1 = OH, R^2 = CO_2H, R^3 = RhR-X
51b3: 5(6)-ROX-RISPC. R^1 = OH, R^2 = CO_2H, R^3 = ROX
51b4: AF647-RISPC. R^1 = OH, R^2 = CO_2H, R^3 = AF647
51c1: 5(6)-FAM-dRIS. R^1 = H, R^2 = $P(O)(OH)_2$, R^3 = FAM
51c2: 5(6)-RhR-dRIS. R^1 = H, R^2 = $P(O)(OH)_2$, R^3 = RhR-X

51d1: 5-FAM-ZOL. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 5-FAM
51d2: 6-FAM-ZOL. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 6-FAM
51d3: AF647-ZOL. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = AF647
51d4: 800CW-ZOL. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 800CW
51d5: Sulfo-Cy5-ZOL. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = Sulfo-Cy5

51e1: 5-FAM-MIN. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 5-FAM
51e2: 6-FAM-MIN. R^1 = OH, R^2 = $P(O)(OH)_2$, R^3 = 6-FAM
51f1: 5-FAM-MINPC. R^1 = OH, R^2 = CO_2H, R^3 = 5-FAM
51f2: 6-FAM-MINPC. R^1 = OH, R^2 = CO_2H, R^3 = 6-FAM

Fig. 17.

Fluorescent bisphosphonate imaging probe “toolkit” derived from clinically used N-heterocyclic BPs (RIS, ZOL, MIN). These probes cover a wider spectroscopic window from visible to near-infrared range, allowing various imaging applications both *in vitro* and *in vivo*.

(Adapted from ref. [109]).

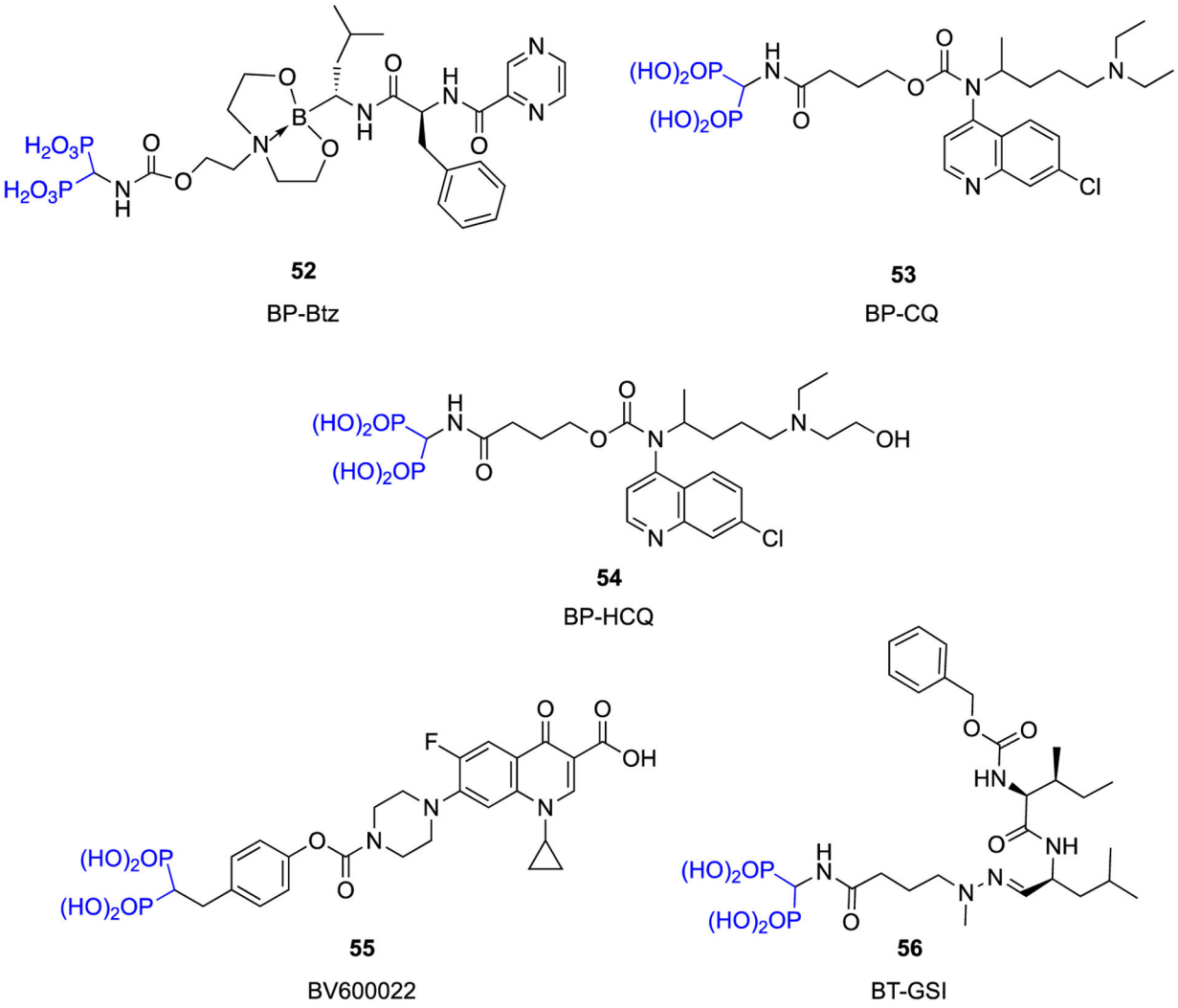
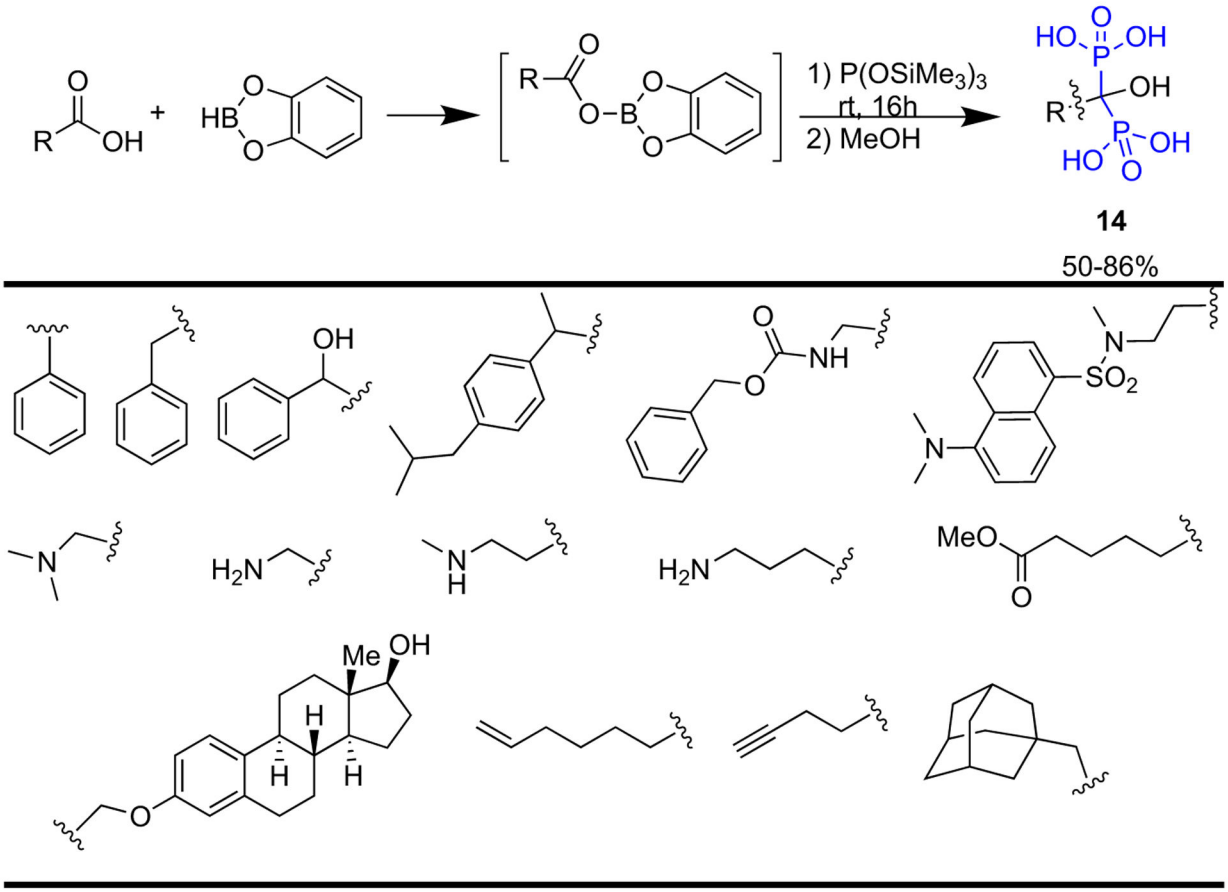
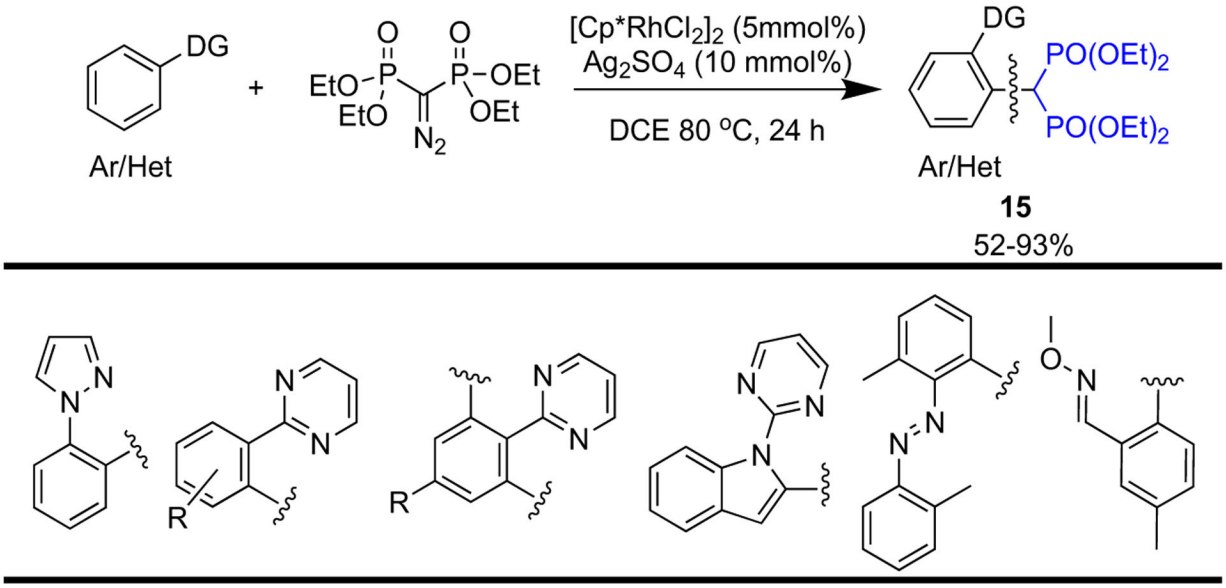


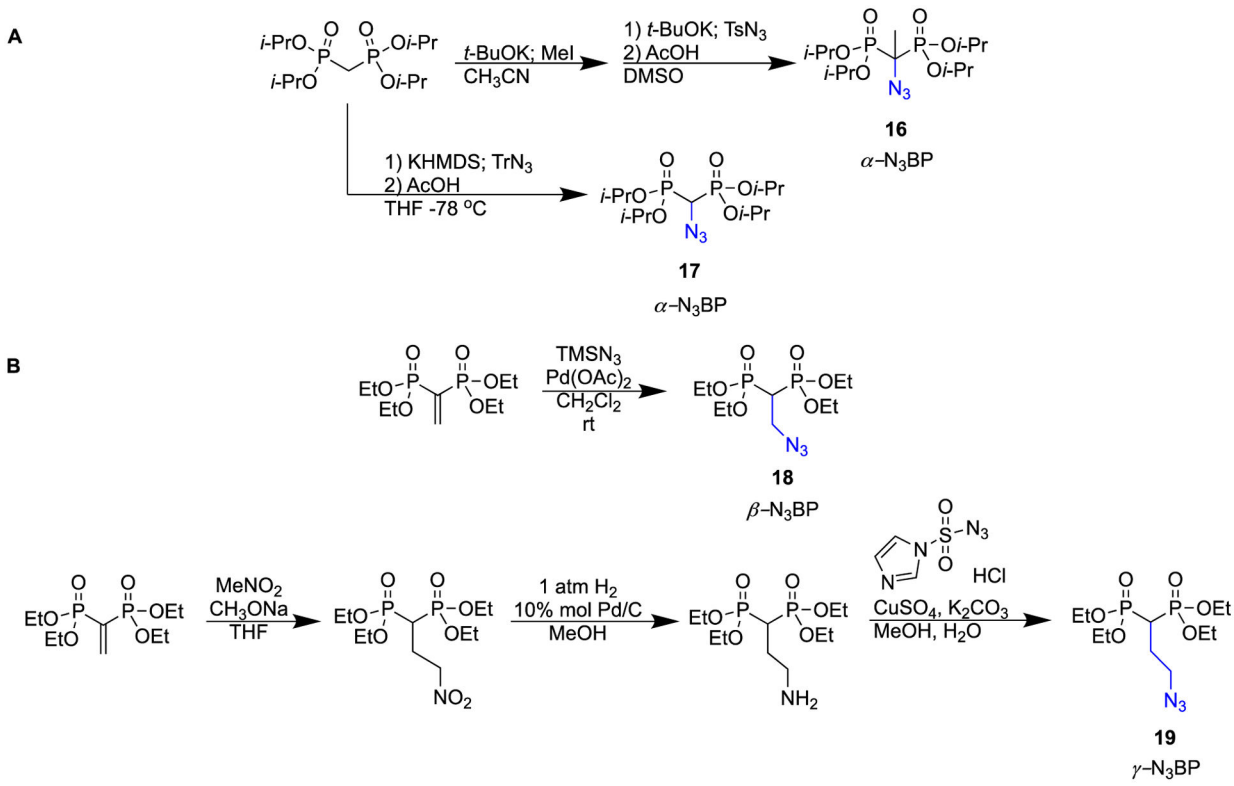
Fig. 18.
Novel conjugates designed based on a “target and release” concept.



Scheme 1.
One-pot synthesis of α-hydroxy bisphosphonates from carboxylic acids.

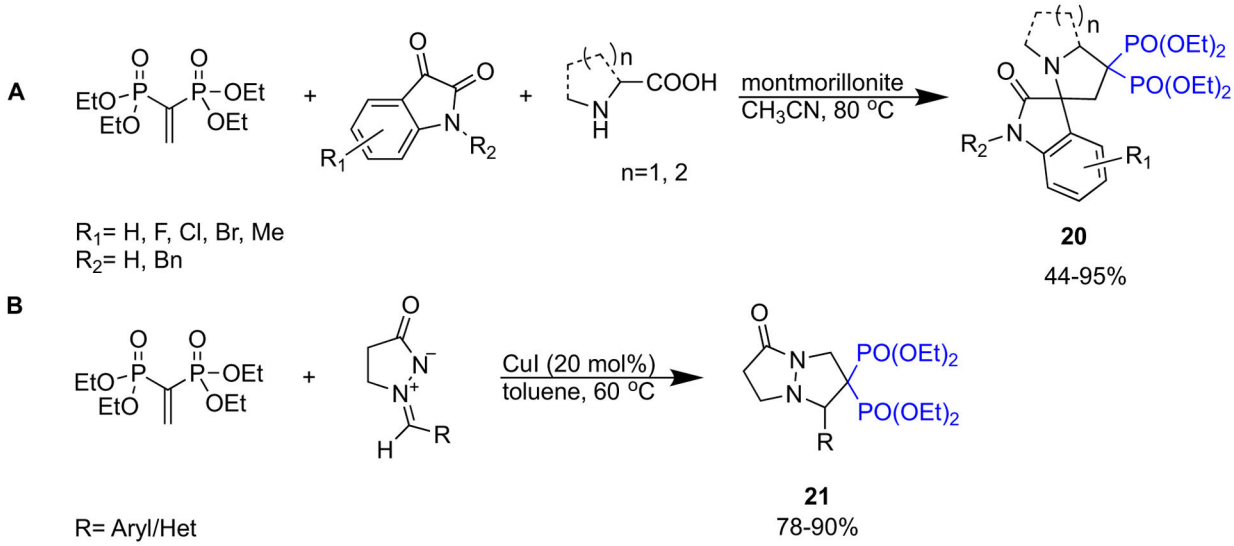


Scheme 2.
One-pot synthesis of aromatic bisphosphonates.



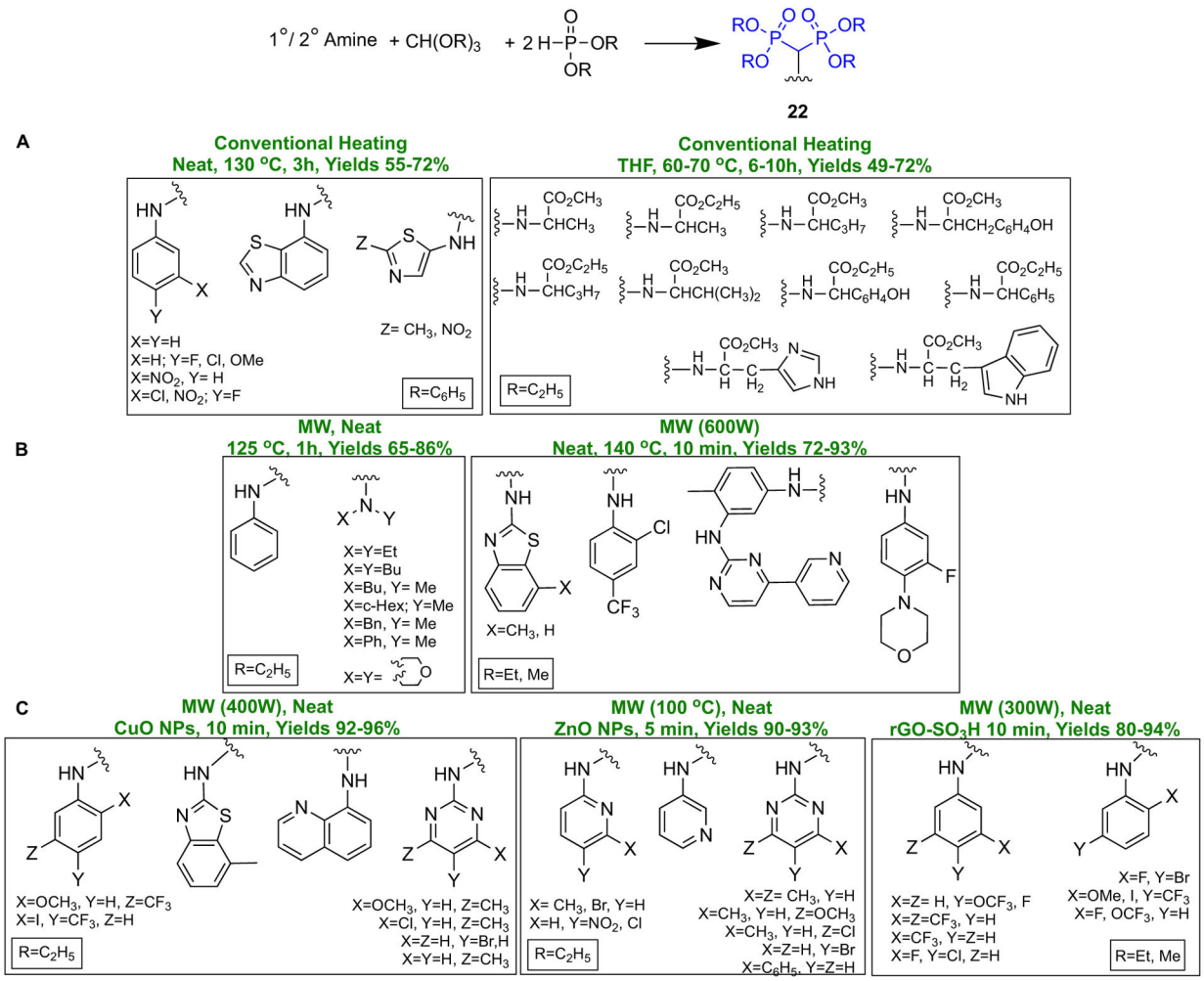
Scheme 3.

Synthetic routes to α-, β-, and γ-azido bisphosphonates.



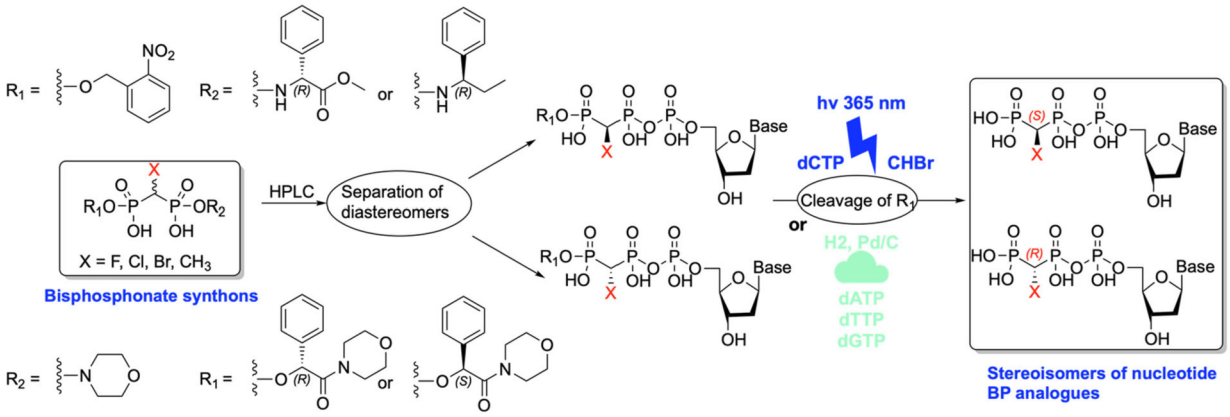
Scheme 4.

Synthesis of spiro bisphosphonates.



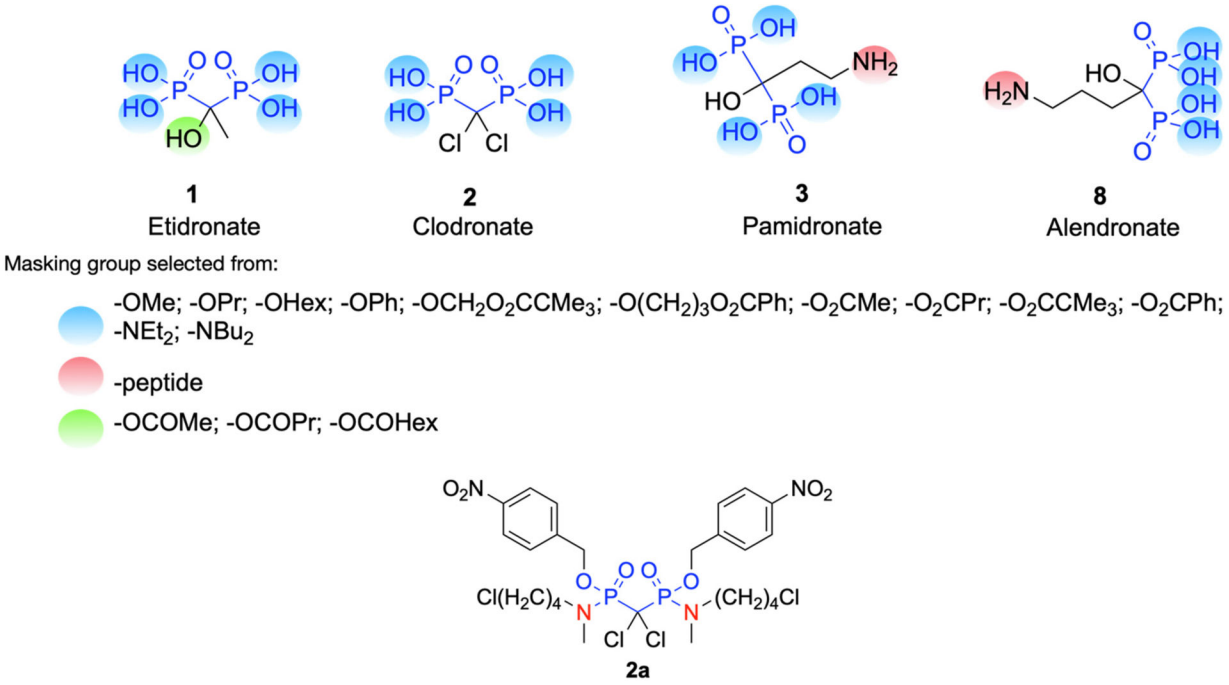
Scheme 5.

Recent methodologies to access α-amino bisphosphonates.



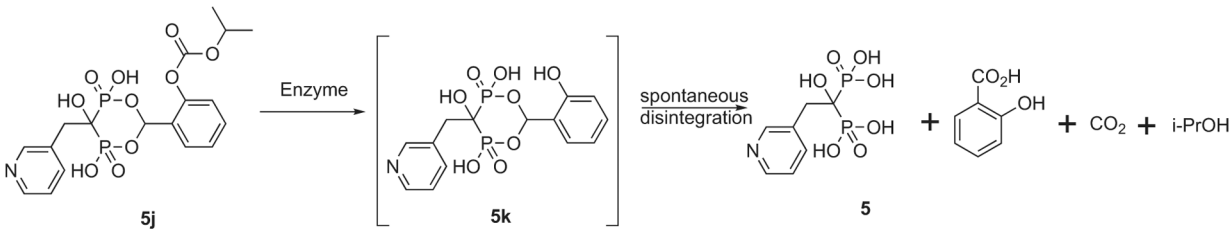
Scheme 6.

BPs used as synthons for preparation of nucleotide analogs.



Scheme 7.

Prodrug design strategies for clinically used bisphosphonates: etidronate, clodronate, pamidronate and alendronate



Scheme 8.

Enzymatic hydrolysis of relatively GI stable cyclic acetal ester of bisphosphonate (**5j**) releases tetracid drugs (**5**) in bloodstream.

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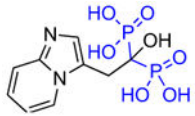
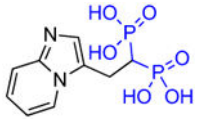
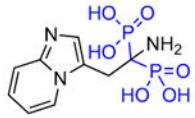
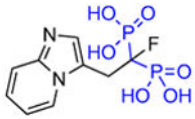
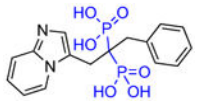
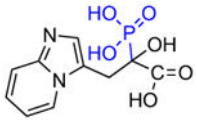
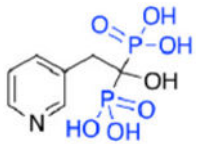
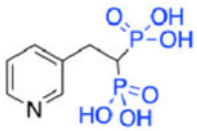
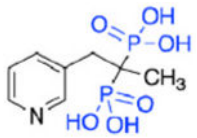
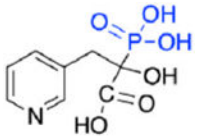
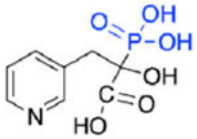
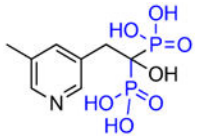
Table 1

Structures of Minodronate and Risedronate analogues with altered substituents and bone mineral affinity leading to the development of OX14 (**10**).

Name	Structure	Inhibition FDPS (IC_{50}, nM)	Inhibition GGPPS (IC_{50}, nM)	Mineral affinity Retention time (min)
Minodronate (**7**)		1.9	869	10.33
Deshydroxy Minodronate OX8 (**7a**)		2.6	>30,000	6.6
OX9 (**7b**)		15.4	>300,000	26.97
OX14 (**10**)		2.5	7345	6.17
OX18 (**7c**)		628	3100	<3
Minodronate phosphonocarboxylate (**7d**)		>60,000	No inhibition	NA
OX139 (**7e**)		6.6	700,000	6.3

Name	Structure	Inhibition FDPS (IC_{50}, nM)	Inhibition GGPPS (IC_{50}, nM)	Mineral affinity Retention time (min)
Risedronate (**5**)		5.7	5675.4	9.97
Deshydroxy Risedronate (NE58043) (**5a**)		34.3	~2000	5.8
OX11 (**5b**)		664	NA	4.6
OX24 (**5c**)		142	>30,000	14.7
Risedronate phosphonocarboxylate, NE10790 (**5d**)		>250,000	No inhibition	NA
OX78 (**5e**)		5	-	9.17

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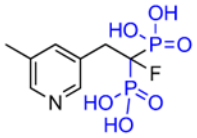
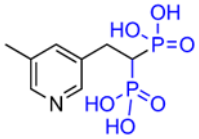
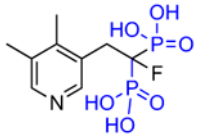
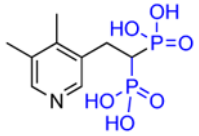
Name	Structure	Inhibition FDPS (IC_{50}, nM)	Inhibition GGPPS (IC_{50}, nM)	Mineral affinity Retention time (min)
OX104 (**5f**)		18	~327,000	5.9
OX105 (**5g**)		13	>750,000	6.1
OX111 (**5h**)		21	120,000	6.3
OX112 (**5i**)		7.4	>340,000	5.9