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REVIEW

# Approaches for Designing new Potent Inhibitors of Farnesyl Pyrophosphate Synthase

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**ABSTRACT**

***Introduction***: Farnesyl pyrophosphate synthase (FPPS) catalyzes the condensation of isopentenyl diphosphate with dimethylallyl diphosphate to give rise to one molecule of geranyl diphosphate, which on a further reaction with another molecule of isopentenyl diphosphate forms the 15-carbon isoprenoid farnesyl diphosphate. This molecule is the obliged precursor for the biosynthesis of sterols, ubiquinones, dolichols, heme A, and prenylated proteins. The blockade of FPPS prevents the synthesis of farnesyl diphosphate and the downstream essential products. Due to its crucial role in isoprenoid biosynthesis, this enzyme has been winnowed as a molecular target for the treatment of different bone disorders and to control parasitic diseases, particularly, those produced by trypanosomatids and Apicomplexan parasites.

***Areas covered***: This article discusses some relevant structural features of farnesyl pyrophosphate synthase. It also discusses the precise mode of action of relevant modulators, including both bisphosphonate and non-bisphosphonate inhibitors and the recent advances made in the development of effective inhibitors of the enzymatic activity of this target enzyme.

***Expert opinion***: Notwithstanding their lack of drug-like character, bisphosphonates are still the most advantageous class of inhibitors of the enzymatic activity of farnesyl pyrophosphate synthase. The poor drug-like character is largely compensated by the high affinity of the bisphosphonate moiety by bone mineral hydroxyapatite in humans. Several bisphosphonates are currently in use for the treatment of a variety of bone disorders. Currently, the great prospects that bisphosphonates behave as antiparasitic agents is due to their accumulation in acidocalcisomes, organelles with equivalent composition to bone mineral, hence facilitating their antiparasitic action.

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## 1. Introduction

Isoprenoids are crucial metabolites of all organisms due to their roles in an assortment of biological events associated with essential cellular processes. These compounds present diverse chemical structures consisting of multiple units of five carbon atoms and are biosynthesized from single precursors: isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP).[1,2] IPP is synthesized through the mevalonate pathway, in which mevalonate is converted into IPP through two consecutive phosphorylation steps and one decarboxylation step. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) is the key regulatory enzyme in this process.[3] Isomerization of IPP catalyzed by IPP isomerase yields DMAPP. In addition, farnesyl pyrophosphate synthase (FPPS), also known as farnesyl diphosphate (FPP) synthase, catalyzes the two consecutive condensations of IPP with DMAPP: the former one produces one molecule of geranyl diphosphate (GPP), which on reaction with another molecule of IPP forms the 15-carbon isoprenoid FPP. This molecule is the obliged precursor for the biosynthesis of sterols, ubiquinones, dolichols, heme A, and prenylated proteins. FPP can undergo a further condensation reaction with an additional molecule of IPP catalyzed by geranylgeranyl pyrophosphate synthase to form the 20-carbon isoprenoid GGPP.[4] Therefore, blockade of FPPS prevents the synthesis of FPP and the mentioned downstream essential products. FPPS has a key role in isoprenoid biosynthesis and, for that reason, this enzyme has been mainly winnowed as a target for different bone disorders such as osteoporosis, Paget's disease, hypercalcemia, tumor bone metastases.[5,6] This enzyme is also the molecular target for other drugs, which act as anticancer agents,[7–9] antibacterial agents,[10] stimulator of γδ T cells in the immune system,[11] and also as antiparasitic agents.[12–17]

The FPPS gene seems to be vital for all organisms.[18,19] FPPSs belong to the prenyltransferase family of proteins, have been characterized as homodimeric enzymes, and require divalent metal ions such as $Mg^{2+}$ or $Mn^{2+}$ for activity.[1] In addition, all of these enzymes show, to a certain extent, a high degree of similarity. In fact, a comparison of the amino acid sequences of FPPSs from different organisms, ranging from bacteria to higher eukaryotes, exhibits the presence of seven conserved regions bearing two aspartate-rich motifs with two binding sites per each individual substrate (IPP/GPP and DMAPP), which are very important for the catalytic activity.[20] Each homodimeric domain has subunit sizes from 32 to 44 kDa.[20]

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Article highlights

- FPPS is present in all organisms and constitutes a key enzyme within the mevalonate pathway and the isoprenoid synthesis.
- FPPS catalyses the condensation between dimethyl allyl pyrophosphate and isopentenyl pyrophosphate to give rise to geranyl pyrophosphate and the subsequent reaction with a further molecule of isopentenyl pyrophosphate to yield farnesyl pyrophosphate.
- FPPS possesses three inhibition binding sites: the allylic site (dimethyl allyl pyrophosphate and geranyl pyrophosphate), the homoallylic site (isopentenyl pyrophosphate), and the allosteric site.
- Bisphosphonate-based inhibitors have been developed as extremely effective inhibitors binding to the allylic site.
- Recently, allosteric inhibitors of drug-like character have been designed and biologically tested.

This box summarizes key points contained in the article

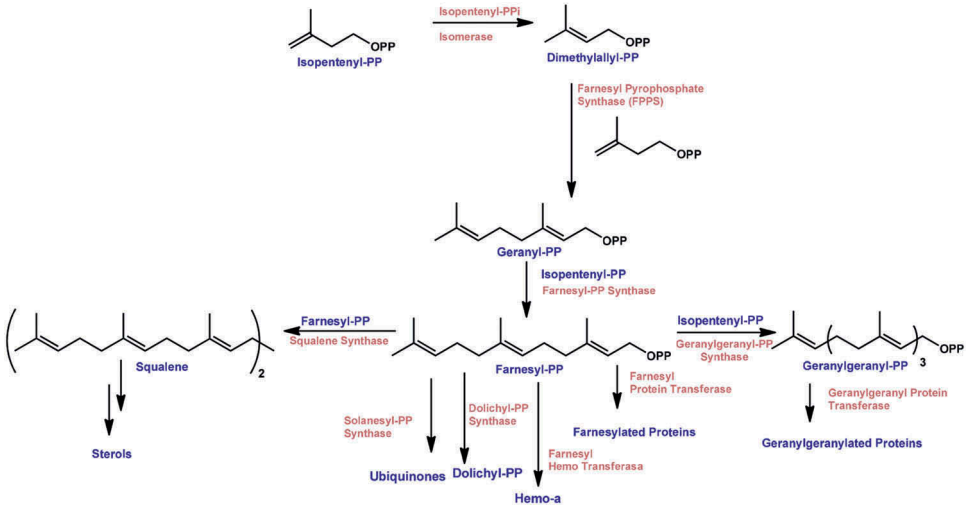
As previously mentioned, FPPS has an important role downstream of isoprenoid biosynthesis. For example, protein prenyltransferases catalyze the transfer of FPP or GGPP to a conserved cysteine residue in the CaaX motif of the protein or peptide substrate.[21] This post-translational modification is essential for a number of proteins including Ras, Rab, nuclear lamins, trimeric G-protein γ subunits, protein kinases, and small Ras-related guanosine triphosphate (GTP)-binding proteins, which are crucial for controlling osteoclast cellular activities.[22] Post-translational modification of proteins with C-15 farnesyl or C-20 geranylgeranyl groups appears to be essential for the localization of these proteins to membranes and hence their biological function.[23–25] Modulators of protein prenyltransferases were developed as new antitumor agents. Additionally, these enzymes were found to be interesting molecular targets in *Plasmodium falciparum*, the responsible agent of malaria, and trypanosomatids.[26] The occurrence of prenylated proteins and farnesyl transferase activity in the hemoflagellated parasite *Trypanosoma cruzi*, the etiologic agent of American trypanosomiasis (Chagas disease), was depicted some years ago.[27–29] The importance of this enzyme is illustrated in Scheme 1.

## 2. Structural features

FPPS was first described in 1959,[30] and due to its key role in essential biosynthetic pathways, the development of FPPS modulators was of paramount relevance. As substrate analogs, bisphosphonates emerge as very interesting inhibitors of the enzymatic activity of FPPS. These compounds were envisioned based on the structure of the natural substrate where the inorganic pyrophosphate (**1**) is converted into a bisphosphonate (**2**) by substitution of the oxygen atom linking the two phosphorous atoms by a methylene group.[31] Further modification of the $R^1$ and $R^2$ groups bonded to the C-1 position can give rise to a wide variety of compounds. The relevance of these drugs started from calcification studies performed several years ago.[32–34] Figure 1 shows the representative bisphosphonates that are currently in clinical use for the treatment and prevention of osteoclast-mediated bone resorption, associated with a number of bone disorders. They are known as etidronate (**3**), clodronate (**4**), pamidronate (**5**), alendronate (**6**), risedronate (**7**), tiludronate (**8**), ibandronate (**9**), zoledronate (**10**), and incadronate (**11**).[5,35–37]

The ability of bisphosphonates to exert their biological action in bone disorders as inhibitors of bone resorption is due to their affinity with bone mineral hydroxyapatite.[38] In addition, there exits some evidence to believe that bisphosphonate can equally regulate osteoblast and osteocyte apoptosis.[39,40]

Certainly, bisphosphonates are very interesting molecules that are metabolically stable substrate analogs. Most bisphosphonates currently employed for the treatment of bone disorders have a hydroxyl group bonded to the C-1 position.[35–37] As an electron withdrawing group, this functionality affects the $pK_a$ values of the phosphonate moieties, that is, the acidity of these functionalities, influencing the ability to coordinate divalent cations such as $Ca^{2+}$ or $Mg^{2+}$.[41–46] Bisphosphonates are not limited to coordinate the above metals, but also $Zn^{2+}$, $Cu^{2+}$, $Fe^{3+}$, and $Al^{3+}$.[43,44,46] Additionally, the nature of the substituent at the C-1 position, particularly if it is an electron withdrawing unit, can increase




Scheme 1. Brief overview of farnesyl diphosphate formation catalyzed by farnesyl diphosphate synthase, leading to crucial metabolites for different organisms.

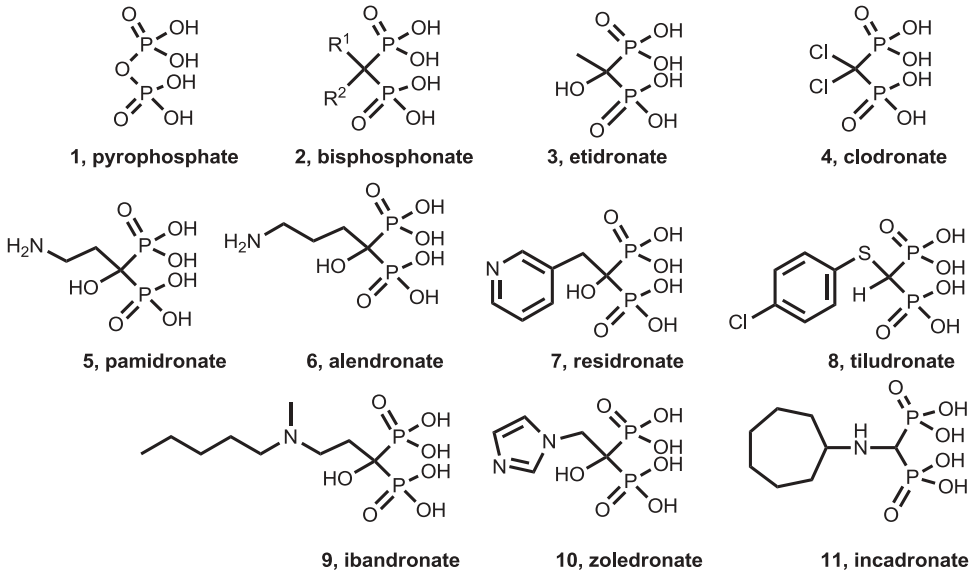



Figure 1. General formula and chemical structure of representative FDA-approved bisphosphonates clinically employed for the treatment of different bone disorders.

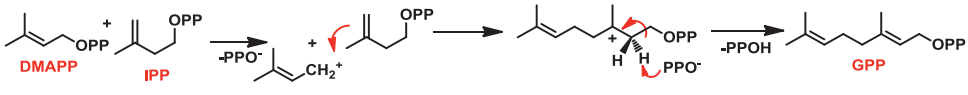


acidity values up to one order of magnitude.[47–52] In general, the selection of the substituent at C-1 is tuned to mimic the p$K_a$ value of pyrophosphoric acid. The structural variations at the C-1 position and in its vicinity have a marked influence in biological activity as will be discussed later. Non-bisphosphonate modulators of FPPS are also available, though to a lesser extent than the bisphosphonate derivatives.[20] In the last years, some review articles have appeared about the scope, modulators, and usefulness of this key enzyme.[20,53–55] One of them is focused on the patent literature of bisphosphonates.[20] The work by Dhar et al. summarizes the literature regarding the role of FPPS within the mevalonate pathway, its characterization, localization, evolution, and gene expression and regulation, concluding that this enzyme has an important pharmaceutical role with bisphosphonates as the main class of inhibitors.[53] The article by Ebetino et al. discusses the structure, chemical synthesis, interactions in the binding site with the receptor residues, and the distribution in bone mineral of bisphosphonates.[54] The latest deals with computation studies modeling the interactions between nitrogen-containing bisphosphonates and FPPS at the active site.[55]

The proposed reaction mechanism for the formation of geranyl pyrophosphate requires the abstraction of the pro-R proton at the C-2 position of the carbenium intermediate (Scheme 2). Although the p$K_a$ of the intermediate is low, it is still necessary to provide a base for the abstraction of the proton and for providing a path to the bulk solvent. This arrangement indicates that in the last step of the reaction, the diphosphate that separates from the second substrate will be ideally positioned to abstract the proton from IPP, as was suggested by the pioneering works of Cornforth *et al*.[56,57] As this pyrophosphate is released, it will carry the abstracted proton to the bulk solvent. Two sequential reactions carried out by FPPS may take place without releasing the product of the first reaction. Further X-ray crystallographic studies were employed to propose the precise mode of action of bisphosphonates targeting FPPS. Once the C–C bond is formed, the pyrophosphate group of DMAPP is released, and the subsequent rearrangements take place. The resulting GPP preserves the positions of the atoms with respect to those of the pre-catalytic complex.[58] Some years ago, it was postulated that nitrogen-containing bisphosphonates, specifically those that have a nitrogen atom at the C-4 position as is the case for risedronate, would act as a carbocation transition state analog of isoprenoid diphosphates.[59] Nevertheless, as will be discussed later, based on the X-ray structure of different complexes bisphosphonates–FPPS, these compounds should instead be considered as competitive inhibitors of the enzymatic activity of FPPS rather than transition state analogs.

Many X-ray crystallographic studies have been conducted in FPPS from a number of organisms, which provide further insights to determine the precise mode of action of bisphosphonate and non-bisphosphonate inhibitors. In fact, at the present time, there are crystallographic data available of this enzyme from: human,[9,60–66] avian,[67,68] *Saccharomyces cerevisiae*,[69] plants,[57] *T. cruzi* (the etiologic agent of Chagas disease or American trypanosomiasis),[70,71] *Trypanosoma brucei* (the responsible agent of sleeping sickness),[72,73] *Leishmania major*,[74,75] *Plasmodium vivax*,[75] *Mycobacterium tuberculosis*,[76] *Escherichia coli*,[77] among other species.

As mentioned before, the enzyme has two regions at the catalytic site: the allylic diphosphate (A-site) and homoallylic




Scheme 2. Proposed reaction mechanism for GPP formation from IPP and DMAPP.

diphosphate (I-site) sites,[57] which can be considered as subdivided into two subsites. The two subsites of the allyl diphosphate binding region are the allylic pyrophosphate (PPAl) subsite, which binds the diphosphate moiety of the acceptor molecule (either DMAPP or GPP), and the Al subsite, which binds the hydrocarbon chain. The subsite PPAl recognizes the diphosphate of the molecule being released as the reaction occurs. This site also contains the three $Mg^{2+}$ ions that take part in the catalysis process.[58] The overall structure of different FPPSs can be described as a two-helix N-terminal hairpin followed by a central eight-helix bundle. The dimeric structure of the complex alendronate–FPPS is illustrated in Figure 2. In fact, superimposition of the crystal structures of enzymes from diverse organisms such as human,[61] *T. cruzi*, [58] avian,[67] and *L. major*,[74] performed using the MultiSeq analysis environment [78] in VMD,[79] shows a high level of similarity as illustrated in Figure 3a. In addition, risedronate is a potent inhibitor of the enzymatic activity of both human FPPS (*h*FPPS) [61] and *T. cruzi* FPPS (*Tc*FPPS).[58] As shown in Figure 3b, at the active site of the enzyme, risedronate occupies the allylic site in a similar way in both species. Interestingly, the $Mg^{2+}$ ions play a key role: as exemplified by the complex risedronate–FPPS, all three $Mg^{2+}$ ions bind to both phosphonate units present at allylic site and no $Mg^{2+}$ ion is associated with the IPP substrate at the homoallylic site. The mechanistic role of phosphate coordination to $Mg^{2+}$ is thought to be their stabilization as leaving groups as the ionization reaction occurs.[58] This observation justifies why $Mg^{2+}$ binding only takes place in the presence of either the natural substrate or the corresponding competitive inhibitor.[80]

The sequence found in *Tc*FPPS is also present in *T. brucei* FPPS (TbFPPS), although its conformation in the X-ray structure appears different. This was originally attributed to the lack of experimental phases in the 3.6 Å resolution *T. brucei* crystal structure.[73] However, the conformational difference between *Tc*FPPS and *Tb*FPPS [81] refers to the 11-residue insertion (residues 179–189 in *Tc*FPPS) that is unique to trypanosomatid FPPSs. As this loop structure is far from the active site and is thought to be mobile in solution, this structural difference is not expected to effect a significant difference in the binding of competitive inhibitors between *Tc*FPPS [58] and *Tb*FPPS [81] as illustrated in Figure 3c. The structural differences at the active site are very minor to justify the selectivity found toward these enzymes among many compounds that modulate their enzymatic activity. It is worth mentioning that FPPS of *Toxoplasma gondii*, the responsible agent of toxoplasmosis, is a bifunctional enzyme that catalyzes the condensation of IPP with three allylic substrates: DMAPP, GPP, and FPP. [82] Interestingly, *T. gondii* FPPS (*Tg*FPPS) has less than 50% sequence similarity with *Tc*FPPS; which could allow the development of highly selective inhibitors toward each enzyme.[12] At the present time, there is no crystal structure of *Tg*FPPS available but the overall structure in the vicinity of the active site is quite similar in comparison with the counterpart enzyme from *P. vivax*.[75] Using homology modeling as implemented in the SWISS-MODEL server, we constructed the 3D-structure of *Tg*FPPS based on the structure of *P. vivax* FPPS, a small enzyme showing the highest degree of similarity with the sequence of *Tg*FPPS, as can be observed in Figure 3d.[83–85] Further illustrating the importance of this family of enzymes, functionally related enzymes catalyzing isoprenoid biosynthesis are found in *M. tuberculosis*.[76,86] The *cis*-type short-chain prenyltransferase Rv1086 catalyzes the formation of *Z,E*-farnesyl-PP, a metabolite that is crucial for cell wall biosynthesis.

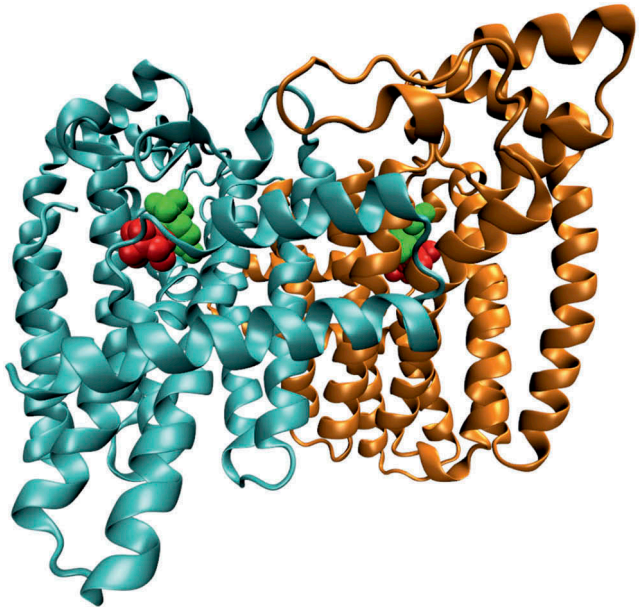


Figure 2. Dimeric structure of the complex alendronate–FPPS (PDB ID 1YHM), alendronate (green), IPP (red).

## 3. Bisphosphonate-based inhibitors

Linear bisphosphonates have become relevant antiparasitic agents, especially 2-alkylaminoethyl derivatives, which were shown to be potent growth inhibitors of amastigotes, the most clinically relevant form of *T. cruzi*, and which were determined to have $IC_{50}$ values in the low nanomolar range against *Tc*FPPS.[87,88] Compounds **12**–**16** come out as representative members of linear bisphosphonates, whose chemical structures are presented in Figure 4. Compounds **12**–**16** inhibit the enzymatic activity of *Tc*FPPS with $IC_{50}$ values of 0.038 μM, 1.84 μM, 0.49 μM, 0.058 μM, and 0.013 μM, respectively.[88] 2-Alkylaminoethyl bisphosphonates have been originally designed to maintain the capacity of bisphosphonate to coordinate $Mg^{2+}$ in a tridentate manner at the active site of *Tc*FPPS and *Tb*FPPS. However, X-ray-crystallography-based studies on the interaction of inhibitor **15** with *Tc*FPPS have indicated that neither the nitrogen atom of the 2-alkylamino moiety nor the hydroxyl group at the C-1 position do in fact form part of the $Mg^{2+}$ ions coordination spheres.[71] The tridentate coordination structure is circumvented to the hydroxyl groups bonded to the phosphorus atoms either for 2-alkylaminoethyl- or 1-hydroxy-1,1-bisphosphonates.[70,72] In addition, the X-ray structure of the complex risedronate–*Tc*FPPS indicates that the Asp250 residue forms a hydrogen bond with the hydroxyl group at the C-1 position of risedronate, which cannot occur with the 2-aminoalkyl derivatives.[58] On the other hand, the effect of the hydroxyl group at C-1 on the

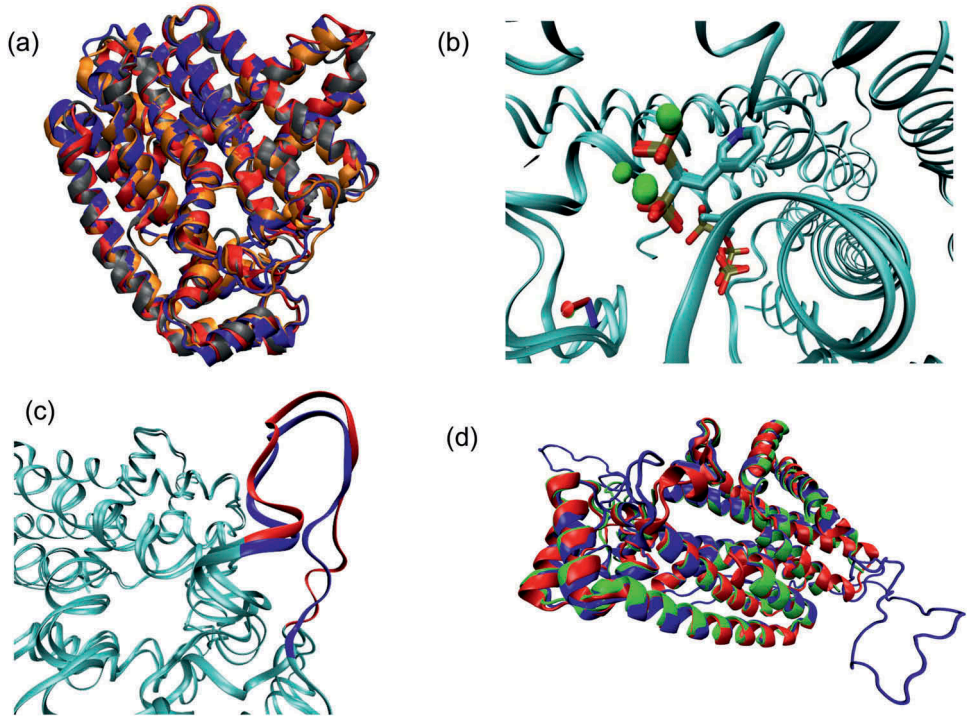
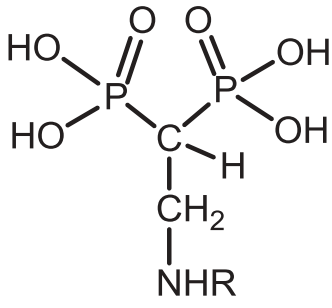



Figure 3. (a) Superposition of different FPPSs: from *T. cruzi* (blue, PDB ID 1YHK), human (red), avian (grey, PDB ID 2F7M), *L. major*: (orange, PDB ID 4JZB); (b) Superposition of the complexes risedronate–*h*FPPS and risedronate–*Tc*FPPS; (c) Superposition of the corresponding crystal structures of *Tb*FPPS (red, PDB ID 4RYP) with *Tc*FPPS (blue); (d) Homology model of *Tg*FPPS built through SwissModel. The structure (blue) is superimposed with FPPS from *P. vivax* (green, PDB ID 3MAV) and *Tc*FPPS (red).




**12**, R = *n*-propyl
**13**, R = *n*-pentyl
**14**, R = *n*-hexyl
**15**, R = *n*-heptyl
**16**, R = cyclohexyl

Figure 4. Chemical structure of representative linear 2-alkylaminoethyl bispohosphonates targeting FPPS.

enzymatic activity of bisphosphonates remains unclear. As the hydroxyl group at C-1 does not actively participate in coordination with $Mg^{2+}$ ions, its role could instead be the modification of the capacity of the adjacent bisphosphonic moiety to chelate $Mg^{2+}$ or to increase the p$K_a$ of the *gem*-phosphonate moiety.

Binding of 2-alkylaminoethyl-1,1-bisphosphonates is not enthalpically favorable.[71] The favorable entropy of the process can be explained as the result of a fine equilibrium between two opposite effects that leads to the required favorable free-energy: the freezing of single bond rotations of the linear bisphosphonate alkyl chain upon binding accounts for the unfavorable loss of conformational entropy, whereas the burial of the alkyl chains is a favorable event form the point of view of solvent entropy.[71] The shortest inhibitor (**12**) produces the tightest binding in this series.[71] It has been proposed that the His93 and Tyr94 residues in *Tc*FPPS form a 'floor' at the allylic site, effectively limiting the maximum length of the inhibitors that can bind to the enzyme.[89] The complexes **13** – *Tc*FPPS, **14** – *Tc*FPPS, and **15** – *Tc*FPPS show that Tyr94 resettle in a different conformation to adjust longer alkyl chains. Increasing the length of the alkyl chain from *n*-propyl (**12**) to *n*-pentyl (**13**) diminishes the affinity dramatically.[71] This structural variation indicates that by the addition of two or three methylene groups, the increase in the loss of conformational entropy is greater than the extra entropy acquired as a consequence of the burial of the longer chain, an effect more noticeable for the pentyl group than for the hexyl group. When the alkyl chain is the *n*-heptyl group, however, the affinity is decreased due to the clashes observed for the end carbon with protein residues Tyr94, His94, or Ile129 in chain B.[71] This observation illustrates the fact that small structural variations have a marked effect on inhibitory action, making simulations for this system very challenging. As can be seen in Figure 5a, a significantly different conformation is observed for **13** compared with **12** and **15**. The phosphate groups from the bisphosphonates directly bind to the three $Mg^{2+}$ ions present in the allylic site, which are arranged in an unsymmetrical

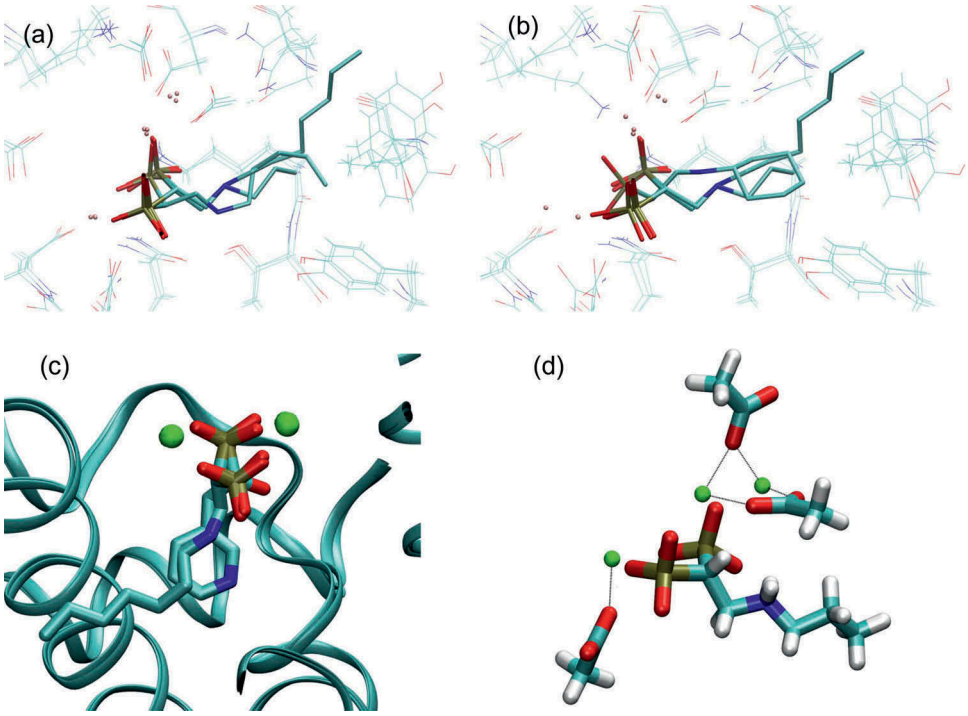



Figure 5. (a) Differing conformations at the binding site of *n*-pentyl *vs* *n*-propyl and *n*-heptyl aminoethyl bisphosphonates; (b) Comparison of cyclohexyl *vs* *n*-propyl and *n*-heptyl aminoethyl bisphosphonates; (c) Superposition of risedronate and **15** bound to *T. cruzi* FPPS; (d) 2-alkylaminoethyl bisphosphonate **12** coordinated with three $Mg^{2+}$ ions at the active site of FPPS. Negative aspartates balance the global charges: Asp 102 (up), Asp98 (right) and As205 (down). Asp 98 is bonded to two $Mg^{2+}$ ions.

fashion since the third $Mg^{2+}$ ion binds exclusively to a single phosphate group (Figure 5b). As a consequence, two conformations are possible for the bisphosphonates in this site: they can either point the C-1 substituent toward or away the third $Mg^{2+}$ ion, and it is expected that the interconversion between these two conformations is slow since it requires the breaking and forming of the strong phosphate–$Mg^{2+}$ interactions. Interestingly, the *n*-pentyl and cyclohexyl aminobisphosphonates (**13** and **16**, respectively) point the C-1 substituent away the third $Mg^{2+}$ ion in the X-ray structures, whereas bisphosphonates **12** and **15** orientate themselves in the opposite fashion. This change brings about a different conformation of the alkyl chain, and especially of the nitrogen atom, so could therefore have a large effect on the binding energy. The binding of the *gem*-phosphonate moiety of risedronate in the complex risedronate–*Tc*FPPS [58] matches the corresponding one of **15** – *Tc*FPPS (Figure 5c). [71] This behavior offers further insights to the precise mode of action of bisphosphonate inhibitors. Supplementary analysis of the X-ray structure of complex **12** – *Tc*FPPS indicates that all the P–O bond distances are equal (1.5 Å) suggesting all oxygen atoms are equivalent and therefore the phosphonates are fully deprotonated. Interestingly, the three coordinating $Mg^{2+}$ ions and the residues Asp 102, Asp 98, Asp 205, and the protonated amino group of the bisphosphonate compensate the global charge yielding a neutral species as illustrated in Figure 5d.

A recent neutron diffraction study has investigated the protonation state and hydration of risedronate bound to *h*FPPS. It was found that the phosphate groups in the bisphosphonate are fully deprotonated in the active site, due to the stabilization of this form provided by the three $Mg^{2+}$ ions and basic amino acid residues present in the allylic site, effectively decreasing the highest p$K_a$ of the bisphosphonate from the 12.0 value determined in solution.[90] In addition, the nitrogen atom in the pyridine ring of risedronate was found to be protonated, even though this nitrogen atom (p$K_a$ 6.1) would be present in the unprotonated form in solution. It is therefore expected that compounds that can stabilize the protonated form would be more effective, as it is the case for the linear nitrogen-containing bisphosphonates.

The hydroxyl group bonded to C-1, which is present in most bisphosphonates clinically in use for the treatment of bone disorders, plays an interesting role in the activity of nitrogen-containing linear bisphosphonates, since the 1-hydroxy analogs of **12**–**16**, that is **17**–**21** (Figure 6), are devoid of activity toward *Tc*FPPS, whereas they are effective inhibitors of *Tg*FPPS.[91] For instance, **20** shows an $IC_{50}$ value of 0.039 μM against *Tg*FPPS. Attempts have been made to explain the loss of activity against *Tc*FPPS for nitrogen-containing bisphosphonates bearing a hydroxyl group at C-1, including a computational study [91] which looks at the formation of an intramolecular hydrogen bond for certain bisphosphonate conformations, which can only occur for 2-alkylamino bisphosphonates, but not for nitrogen-containing bisphosphonates having the nitrogen atom at different positions, such as risedronate and compound **22**, both effective *Tc*FPPS inhibitors.[92,93]

On the other hand, evaluation of linear α-fluoro-1,1-bisphosphonic acids illustrates the different activities and therefore high selectivity among FPPSs. Undoubtedly, the presence of the electron-withdrawing fluorine atom influences the ability to coordinate to $Mg^{2+}$.[48–51] Fluorine-containing

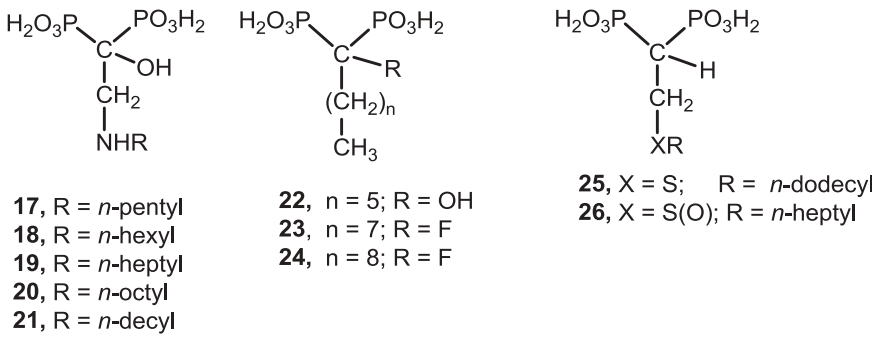



Figure 6. Chemical structure of representative linear amino- fluorine- and sulfur-containing ispohosphonates targeting FPPS.



bisphosphonates **23** and **24** are important derivatives of this type of compounds. Although **23** and **24** are devoid of activity against *Tc*FPPS, they work as nanomolar inhibitors toward *Tg*FPPS, exhibiting $IC_{50}$ values of 0.035 μM and 0.060 μM, respectively.[94] Linear sulfur-containing bisphosphonates such as sulfide **25** and sulfoxide **26** are also potent inhibitors of *Tg*FPPS, bearing $IC_{50}$ values of 0.021 μM and 0.009 μM, respectively.[95] Both of these compounds are devoid of biological activity toward *Tc*FPPS.[95]

Although there are abundant inhibitor–protein complexes characterized by X-ray crystallography, sometimes the corresponding enzymatic inhibition data are missing, which on our opinion are relatively straightforward to obtain. We would like to stress here the importance of publishing biological activity data alongside the X-ray structures to be able to fully understand the binding of inhibitors to the target enzyme, particularly for this challenging system.

The effective bisphosphonate inhibitors can be classified as slow, tight-binding inhibitors,[96] that is, binding equilibrium is slowly attained probably due to the protein conformational changes required for better molecular fitting.[97] In fact, it has been known for many years that alendronate is a nanomolar inhibitor of *h*FPPS.[98] For instance, zoledronate and risedronate show initial $IC_{50}$ values of 0.475 μM and 1.079 μM against *h*FPPS, respectively, but final $IC_{50}$ values of 0.0041 μM and 0.0057 μM, respectively.[99] Compounds **27**–**29** (known as NE58018, NE97220, and NE58062) are also relevant examples of bisphosphonates that behave as slow, tight-binding inhibitors of *h*FPPS. They show initial $IC_{50}$ values of 0.349 μM, 0.0752 μM, and 0.541 μM and final $IC_{50}$ values of 0.0092 μM, 0.0062 μM, and 0.0103 μM, respectively.[99] The chemical structures of these compounds are illustrated in Figure 7.

It is worth mentioning the case of conformationally constrained bisphosphonates such as enantiomers **30** and **31**. In fact, it was found that the spatial orientation has a marked influence on inhibitory action being only one isomer responsible of the biological activity. Compound **30** ((1*R*,6*S*)-2-azabicyclo[4.3.0]nonane-8,8-bisphosphonate; PG-1014491) is undoubtedly the actual inhibitor ($IC_{50}$ of 15 nM) compared to its enantiomer (1*S*,6*R*) **31** (PG-1014493), which shows an $IC_{50}$ of 359 nM.[60,100,101] It has been suggested that the nitrogen atom of **30** (Figure 7) has the proper orientation to interact with residues Thr 201 and Lys 200 via hydrogen bonds.[60,100] X-ray studies on the effect of Thr 201 and Tyr 204 (PDB ID 4KPD) on the binding affinity of nitrogen-containing bisphosphonates with *h*FPPS, using enzyme mutants, indicate that Thr 201 forms a hydrogen bond with the nitrogen atom of risedronate. The stabilization achieved by this hydrogen bond is found to be stronger for zoledronate, possibly due to the fact that, based on the $pK_a$'s, the nitrogen atoms are expected to be protonated in zoledronate but deprotonated in risedronate.[101] The nitrogen atoms in pamidronate and alendronate were found to be too far away from the Thr 201 residue to be able to form a hydrogen bond, and mutation of this residue did not affect the inhibitory activity.[101]

Zoledronate derivatives are extremely potent inhibitors of the enzymatic activity of *h*FPPS, particularly those that bear a phenyl group linked to the imidazole ring by a number of methylene units as illustrated by compounds **32**–**34** (Figure 7).[102] These bisphosphonate derivatives exhibit a very potent inhibitory action against the target enzyme (*h*FPPS), possessing $IC_{50}$ values of 0.0019 μM, 0.0014 μM, and 0.0019 μM, respectively.[102]

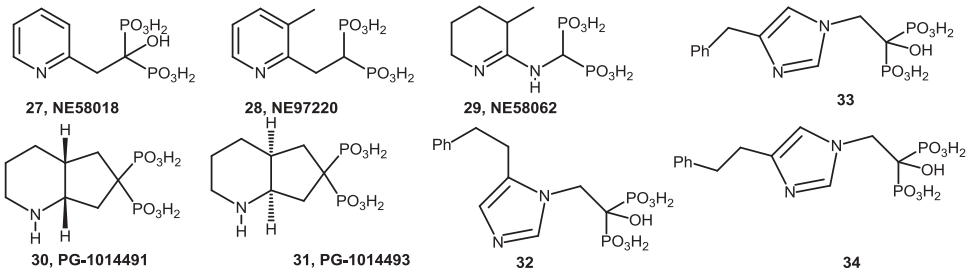



Figure 7. Chemical structure of bisphosphonates that behave as slow, tight-binding inhibitors of human FPPS.

New bisphosphonate bearing isoprenoid alkyl chains have been recently envisioned as inhibitors targeting multiple enzymes within the mevalonate pathway. They act mainly against mammalian FPPS but also toward mevalonate kinase, phosphomevalonate kinase, and mevalonate 5-diphosphate decarboxylase, all of them located downstream from HMG-CoA reductase.[103] The representative compounds **35** and **36** are shown in Figure 8 and they exhibit $IC_{50}$ values of 0.035 μM and 0.029 μM, respectively.[103]

An interesting new class of bisphosphonate-based inhibitors has been recently developed where a hydroxyl group of one phosphonate moiety is replaced by a geranyl skeleton.[104] It is important to note that these nitrogen-containing derivatives are structurally related to other potent inhibitors of *Tc*FPPS as it is the case with **12**–**16**,[87,88] as they have a nitrogen atom at the same position of the aliphatic chain. Compounds **37** and **38** arise as relevant derivatives exhibiting a highly potent inhibitory action against the target enzyme with $IC_{50}$ values of 0.0078 μM and 0.013 μM, respectively.[104] Authors conjecture that these compounds might interact with both the allylic and homoallylic binding site.[104] Their chemical structures are illustrated in Figure 9. Optimizing the structures of these lead compounds could provide a new route for the design of new potent inhibitors, which would utilize both the allylic and homoallylic sites. Designing bisphosphonate-based inhibitors targeting the homoallylic site exclusively, however, is not expected to yield potent compounds, since they would miss the extra stabilization energy provided by the $Mg^{2+}$ ions in the allylic site.

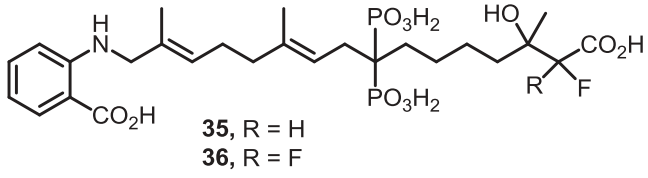



Figure 8. Chemical structures of isoprenoid bisphosphonates targeting multiple enzymes in the mevalonate pathway.

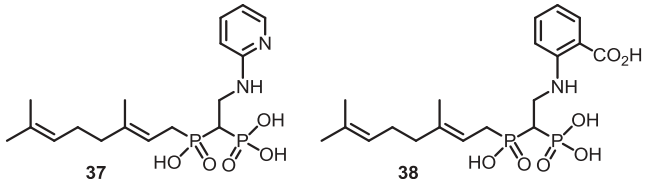



Figure 9. Chemical structures of new bisphosphonate derivatives where a hydroxyl group of one bisphosphonate has been replaced by a geranyl group.

## 4. Synthesis of bisphosphonates

One of the advantages of the envisioned bisphosphonates is that they are straightforward and relatively inexpensive to synthesize. For example, 1-hydroxy-1,1-bisphosphonates of general formula **39** have been prepared using an inexpensive and reliable method developed by Kieczykowski et al. first published in 1995.[105] The common synthetic precursor for this protocol is a carboxylic acid of formula **40**, as shown in Scheme 3. Another reliable method is to start from the acyl chloride **41**, which is converted into the corresponding α-ketophosphonate **42** through a transformation known as Michaelis–Arbuzov reaction.[106] Further treatment with diethyl phosphite (Pudovik reaction) [107] affords the immediate precursor **43**, which on hydrolysis by treatment with bromotrimethylsilane followed by digestion with methanol gives rise to the title compound **39**.[108] Imidazole [109] or pyridinium perchlorate [110] can be used as catalysts for this reaction.

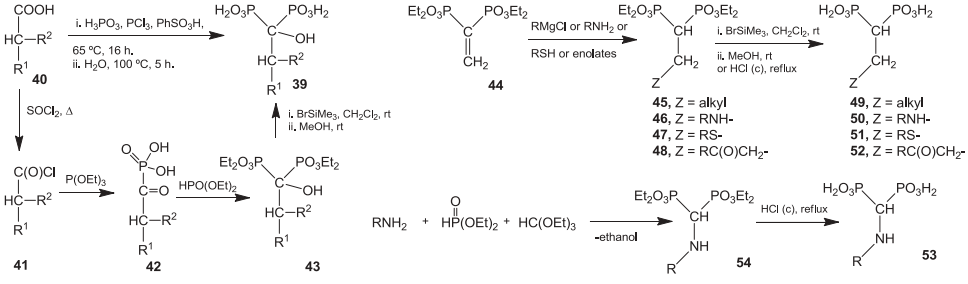
1-Alkyl-1,1-bisphosphonic acids of general structure **49**, 2-alkylaminoethyl-1,1-bisphosphonates **50**, 2-(alkylthio)ethyl-1,1-bisphosphonates **51**, and others (**52**) can be reliably synthesized employing tetraethyl vinylidenebisphosphonate (**44**),[111] which acts as a Michael-type acceptor, giving rise to a variety of bisphosphonates. **44** is readily prepared from commercially available tetraethyl (methylene)bisphosphonate in two steps.[112,113] The Michael adducts **45**–**48** can be hydrolyzed by treatment with bromotrimethylsilane [108] except for the amino intermediates of formula **46**, which should be hydrolyzed by treatment with refluxing hydrochloric acid.[114]

On the other hand, alkylaminomethane-1,1-bisphosphonates of formula **53** can be readily prepared by treatment of the corresponding amine with diethylphosphonate and triethyl orthoformate as illustrated in Scheme 3.[115]

## 5. Allosteric inhibitors

There are many interesting examples of effective inhibitors lacking the *gem*-phosphonate moiety, which act as allosteric inhibitors.

Quinoline derivatives are considered as allosteric inhibitors. Recently a new series of these compounds has been synthesized and evaluated against the target enzyme.[116] From 20 compounds, all of them show a modest micromolar activity toward




Scheme 3. Different synthetic approaches to access a variety chemical structures of bisphosphonates.

FPPS, being **55**–**57** the more effective ones having $IC_{50}$ of 3.5 μM, 3.5 μM, and 3.7 μM, respectively (Figure 10). Docking studies of these quinoline derivatives suggest that they target the allosteric binding site of the enzyme.[116]

In addition, Lindert et al.,[117] using docking studies, identified a lead structure (compound **58**) with a weak inhibitory action against the target enzyme ($IC_{50}$ value of 109 μM). Its structure was used to find similar compounds from the National Cancer Institute database. Based on the similarity search, 10 compounds were found to have modest $IC_{50}$ values in the low micromolar range. The structures of the most relevant compounds such as **59** and **60** ($IC_{50}$ values of 1.8 μM and 1.9 μM, respectively) are shown in Figure 11.[117] The X-ray crystal structure of **59** bound to *h*FPPS has been published: the compound interacts with what has been named as site 4 (S4), close to the allosteric binding site, but which could also be interpreted as a section of the protein surface close to the allosteric binding site.[81]

Salicylic acid derivatives have proven to be efficient allosteric inhibitors of FPPS.[118] New inhibitors were found using fragment-based approaches, and X-ray studies showed that they bind to the allosteric site. A detailed and insightful characterization of this site was carried out, and further structure modification and X-ray studies allowed for an optimization of the inhibition activity, with the strongest binding compound (**61**) having an $IC_{50}$ value of 0.08 μM (Figure 12).[118] The mechanism of inhibition was proposed to involve an impediment of the open to closed conformational transition of the enzyme, necessary for its catalytic activity, and a steric repulsion between the negatively charged allosteric ligands and IPP.[118]

Very recently, the discovery of new allosteric inhibitors of FPPS by 'integrated lead finding' strategy has been depicted.[118,119] The title compounds target the allosteric binding site close to the homoallylic site, and they are based on two

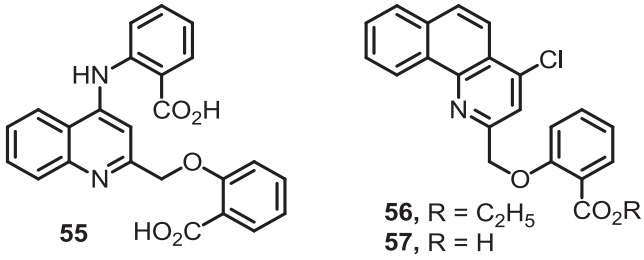



Figure 10. Chemical structures of representative quinoline derivatives, which acts as allosteric inhibitors towards FPPS.

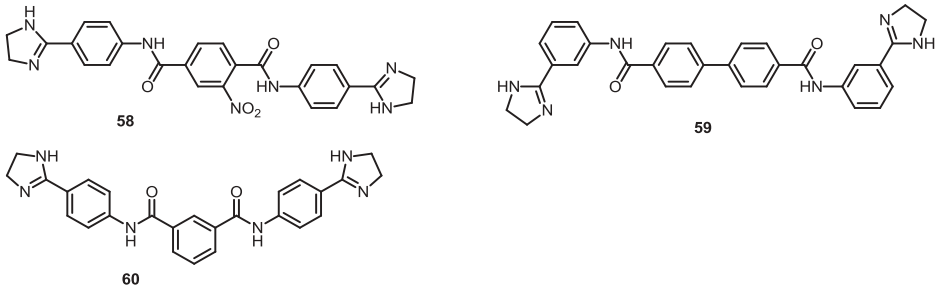



Figure 11. Chemical structures of non-bisphosphonate inhibitors towards FPPS.

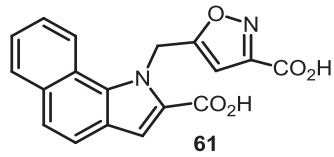



Figure 12. Chemical structure of a highly efficient allosteric inhibitor of FPPS.

different types of inhibitors: salicylic acid and quinoline derivatives, with representative compounds **62** ($IC_{50}$ of 0.038 μM) and **63** ($IC_{50}$ of 0.024 μM), respectively (Figure 13). X-ray studies were performed, showing that the compounds bind to the previously reported allosteric binding site, with the enzyme in the open state and the flexible C-terminal tail disordered (Figure 14).[60] ITC experiments showed that the binding of **64** is enthalpically driven (−8.03 kcal/mol), with a favorable entropic contribution (+3.10 kcal/mol). The carboxylic acid group is crucial for binding, since the allosteric site is abundant in basic amino acids. However, this negatively charged group was thought to account for the low membrane permeability and therefore low cellular activity of the compounds. Attempts to replace the carboxylic group by a tetrazole unit rendered the compounds devoid of inhibitory action.[119]

In addition, new allosteric inhibitors of FPPS were developed by modifying previously reported benzoindole, salicylic, and quinoline derivatives where the corresponding carboxylic acid groups were replaced by a phosphate moiety.[120] This modification increases the bone affinity but has a vanishing effect on the inhibitory action against the target enzyme.[120] X-ray studies showed that the compounds keep binding to the same allosteric site close to the homoallylic site. Figure 15 shows relevant compounds from each of the allosteric inhibitors series: benzoindole (**65**), salicylic (**66**), and quinoline (**67**) derivatives, exhibiting $IC_{50}$ values of 0.4 μM, 0.55 μM, and 0.04 μM, respectively.[120]

## 6. Conclusions

In summary, FPPS is an important enzyme in the isoprenoid biosynthesis pathway, catalyzing the formation of farnesyl pyrophosphate. This is a crucial metabolite for a number of important processes of the cellular machinery. Consequently, its blockage affects a number of biological events that are associated with cell growth and development. As FPPS seems to be essential for all organisms, this enzyme has been selected as a molecular target for drug design in the context of several diseases.

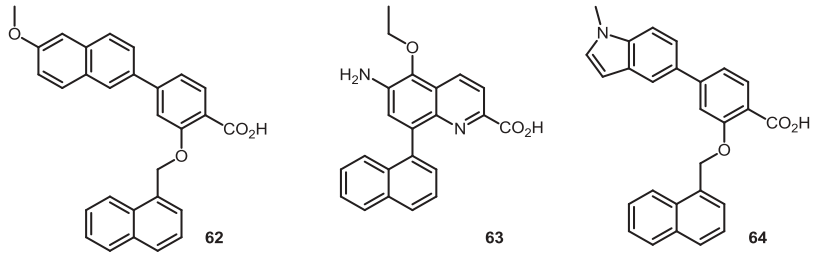



Figure 13. Chemical structures of salicylic acid derivatives targeting FPPS.

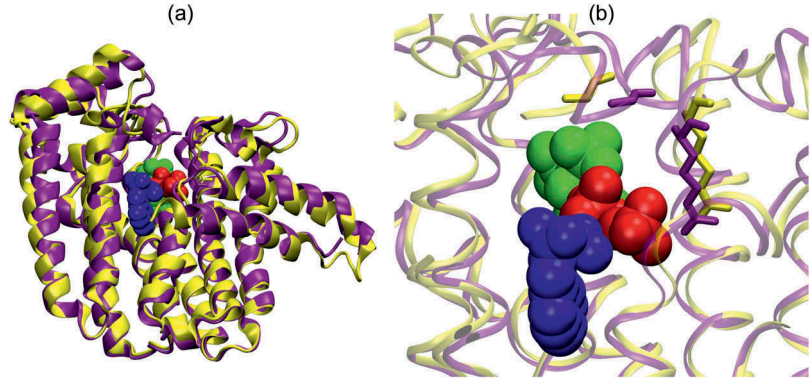



Figure 14. (a) Image showing the superposition of *h*FPPS bound to risedronate (violet, PDB ID 1YV5) and bound to an allosteric inhibitor from the benzoindole series (yellow, PDB ID 3N6K). The enzyme crystallizes in the closed conformation with risedronate, with the Arg127 and Gly270 at the gate of the homoallylic site separated by 4.3 Å (between alpha carbons), whereas it crystallizes in the open conformation with the allosteric inhibitor, showing a corresponding Arg-Gly distance of 8.7 Å. The ligands are shown in VDW representation to highlight the different binding sites: allylic (green), homoallylic (red) and blue (allosteric). (b) Expansion showing the Gly and Arg residues at the gate of the homoallylic site in the open (yellow) and closed (violet) enzyme conformations.

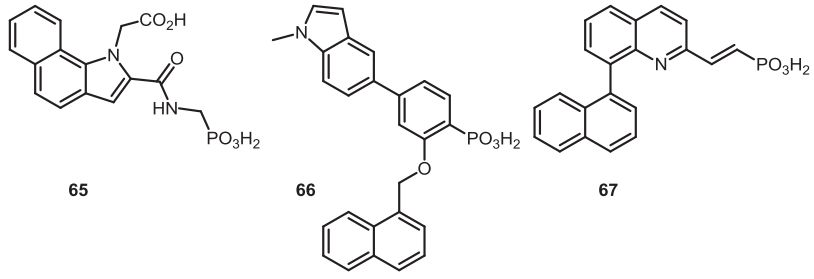



Figure 15. Chemical structures of phosphonated benzoindole, salicylic and quinoline derivatives targeting FPPS.

Three key binding sites have been studied: the allylic site (where dimethyl allyl pyrophosphate or geranyl pyrophosphate bind), the homoallylic site (isopentenyl pyrophosphate), and the allosteric site. All bisphosphonates developed so far interact with the allylic site and no compounds have been developed targeting the homoallylic site. Recently, allosteric inhibitors have been designed targeting the third binding site. For this target, there are many examples where small structural variations on known inhibitors bring about a dramatic effect on biological activity. Therefore, many questions still remain regarding the structure–activity relationships FPPS of inhibitors.

## 7. Expert opinion

FPPS is a key enzyme present in all species. Therefore, it can be selected as a target for a variety of unrelated diseases including different osteoclast-mediated bone disorders, cancer, and the most relevant parasitic diseases, which have a profound social impact worldwide. The target enzyme possesses three binding sites, the homoallylic, the allylic, and the allosteric sites. Bisphosphonate-based inhibitors target the allylic site and, more recently drug-like compounds have been developed targeting the allosteric site. To the best of our knowledge, no inhibitors utilizing the homoallylic site have been published so far.

Notwithstanding their lack of drug-like character, bisphosphonates are still the most advantageous class of inhibitors of the enzymatic activity of FPPS, a key enzyme involved in isoprenoid biosynthesis. The poor drug-like character is largely compensated by the high affinity of the bisphosphonate moiety with hydroxyapatite in human bone mineral. The great prospects that bisphosphonates have as antiparasitic agents is due to the existence of the acidic organelles known as the acidocalcisomes,

which are equivalent in composition to the bone mineral; thus, accumulation of bisphosphonates in these organelles, as they do in bone mineral, facilitates their antiparasitic action.

Bisphosphonates act as metabolically stable competitive inhibitors of the allylic substrate. Small structural variations have a profound effect on the enzymatic activity allowing for a high selectivity toward FPPSs from different organisms.

In spite of there being abundant structural biology data, the challenge of connecting the chemical structure of the inhibitors to their biological activity still remains. Certainly, it is of vital importance for rational drug design to understand the precise mode of action of each inhibitor in the target enzyme. In this sense, several crystal structures of FPPS–inhibitor complexes have been published, either of competitive slow-tight binding inhibitors or the relatively new potent allosteric inhibitors.

This information is very useful for explaining the mechanism of action of the inhibitors, especially since small structural modifications have a large effect on inhibitory activity in this system. However, on many occasions the $IC_{50}$ data for the crystallized inhibitors is missing, making the crystallographic data less useful for establishing structure–activity relationships. Therefore, we believe that the publication of the missing inhibition data would be an important contribution to this field, especially since the assays are relatively straightforward to perform compared to X-ray studies.

For the bisphosphonates, small structural modifications have a large impact on inhibition activity. Furthermore, regardless of the high similarity between FPPS enzymes from different species, several structural variations offer a marked selectivity between species, making these compounds very useful for example as antiparasitic agents since the target enzyme is also present in the host.

Inhibitors of FPPS such as bisphosphonates have been used as first-line drugs in the clinic for the treatment of osteoclast-mediated bone mass loss as a result of osteoporosis, Paget disease, bone metastasis, and other bone disorders many years from now. Moreover, their effectiveness toward parasitic FPPS gives these compounds great potential to become drugs candidates against several parasitic diseases caused by trypanosomatids and Apicomplexans such as Chagas disease, leishmaniasis, sleeping sickness, toxoplasmosis, malaria, among others.

## Financial & Competing Interests Disclosure

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