Bioorganic & Medicinal Chemistry Letters 25 (2015) 1117–1123

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Probing the molecular and structural elements of ligands binding to the active site versus an allosteric pocket of the human farnesyl pyrophosphate synthase

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ARTICLE INFO

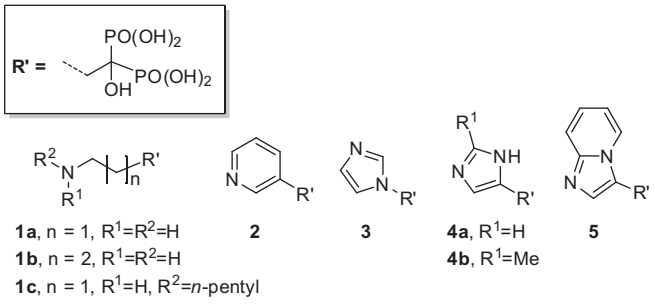
Article history:
Received 17 October 2014
Revised 26 December 2014
Accepted 29 December 2014
Available online 13 January 2015

Keywords:
Bisphosphonates
Human FPPS
Allosteric inhibitors

ABSTRACT

In order to explore the interactions of bisphosphonate ligands with the active site and an allosteric pocket of the human farnesyl pyrophosphate synthase (hFPPS), substituted indole and azabenzimidazole bisphosphonates were designed as chameleon ligands. NMR and crystallographic studies revealed that these compounds can occupy both sub-pockets of the active site cavity, as well as the allosteric pocket of hFPPS in the presence of the enzyme's Mg^{2+} ion cofactor. These results are consistent with the previously proposed hypothesis that the allosteric pocket of hFPPS, located near the active site, plays a feed-back regulatory role for this enzyme.

Bisphosphonates (BPs) are chemically stable bioisosteres of inorganic pyrophosphate that were initially developed as antiscaling, anticorrosion and water softening agents.[1,2] The subsequent discovery that BPs can also prevent bone loss in vivo opened the door to the development of therapeutic agents for the prevention of osteoclast-mediated bone diseases. The most effective of the clinically validated compounds are the nitrogen-containing BPs (N-BPs) having an aliphatic [e.g., pamidronic acid (**1a**), alendronic acid (**1b**), ibandronic acid (**1c**)] or a heterocyclic side chain, such as risedronic acid (**2**), zoledronic acid (**3**) and minodronic acid (**5**). The key biological target of N-BPs is the human farnesyl pyrophosphate synthase (hFPPS),[3] the enzyme which catalyzes the sequential condensation of isopentenyl pyrophosphate (IPP) with dimethylallyl pyrophosphate (DMAPP) to give geranyl pyrophosphate (GPP), and then catalyzes a second condensation between GPP and IPP to form farnesyl pyrophosphate (FPP). Given the strategic location of hFPPS in the mevalonate pathway, this enzyme also plays a pivotal role in controlling the intracellular levels of all human isoprenoids, as well as numerous other essential metabolites. [4]



1a, n = 1, R^1=R^2=H
1b, n = 2, R^1=R^2=H
1c, n = 1, R^1=H, R^2=*n*-pentyl

2 **3** **4a**, R^1=H **4b**, R^1=Me **5**

The key pharmacophore of the current N-BP drugs is their Cα-hydroxyl bisphosphonate moiety (R′). This moiety acts as a tridentate ligand for Mg^{2+} ions in the active site of hFPPS, as well as for Ca^{2+} ions and hydroxyapatite in bone. Since removal of the Cα-hydroxyl usually leads to decreased affinity for bone, this substituent is commonly referred to as the 'bone hook'.[5] The nitrogen on the side chain of N-BPs also plays a role in the activity of these compounds. It is presumed to be protonated and participating in bifurcated hydrogen bond interactions with the side chain hydroxyl of Thr 201 and the carbonyl oxygen of Lys 200 within the hFPPS active site.[3] These interactions mimic those of the putative transition-state allylic carbocation formed during the hFPPS catalytic cycle.[6] N-BPs with an imidazole ring as part of their side chain

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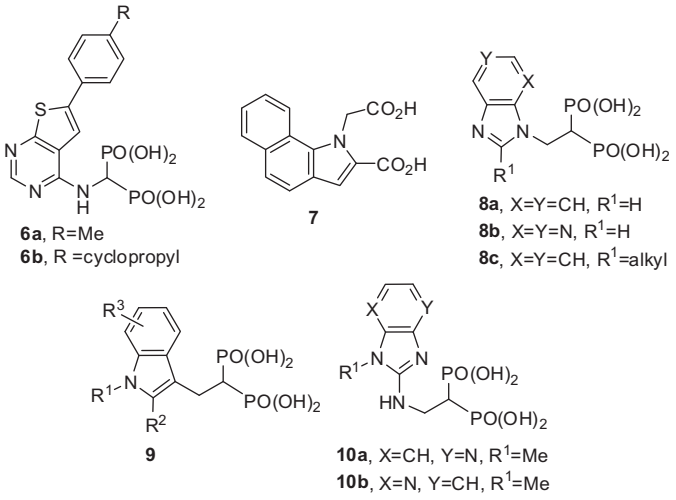
http://dx.doi.org/10.1016/j.bmcl.2014.12.089
0960-894X/

(i.e., **3**, **4**, **5**) are amongst the most potent inhibitors of hFPPS, as well as the most effective therapeutics in blocking osteoclastic bone resorption. Earlier structure–activity relationship (SAR) studies at Ciba-Geigy (now Novartis) explored the direction of attachment from the imidazole to the bisphosphonate (e.g., **3** vs **4**), as well as the influence of substitution on the potency of these compounds.[7] A linker of one methylene unit between the imidazole and the Cα of the bisphosphonate, together with direct attachment of the imidazole nitrogen to the methylene linker was shown to provide optimum potency in reducing hypercalcemia (i.e., blood levels of Ca^{2+}) in rat. For example, the drug Zometa® (**3**) is approximately 4-fold more potent in vivo than analogs **4a** (i.e., EC_{50} difference reflects the dose of compound administered subcutaneously, which results in a 50% reduction of hypercalcemia in rat). Interestingly, although the methyl derivative **4b** is 50-fold less potent than **4a** and >200-fold less potent than **3**, the fused imidazole derivative **5** is nearly equipotent to **3** both in vitro and in vivo. The IC_{50} values in inhibiting hFPPS of **3** and **5** are 4.1 nM and 1.9 nM, respectively, with a minimum effective dose (MED) of 0.003 mg/kg (s.c. administration) in reducing hypercalcemia in rat.[8] A number of structurally related analogs of **5** and their corresponding azaindoles are also potent inhibitors of hFPPS.[9] Consequently, we selected the structurally related fused bicyclic imidazole (**8c**), indole (**9**) and azabenzimidazole (**10**) scaffolds for the design of compounds used in the structural investigations, described in this study.



The interactions between N-BPs and their primary biological target, hFPPS, have been extensively characterized by X-ray crystallography.[3] Numerous structures of hFPPS/ligand complexes have been reported, including the binary complex of hFPPS/**2** (PDB codes: 1YQ7, 1YV5) and the ternary complex of hFPPS/**3**/IPP (PDB code: 1ZW5). These studies have revealed that in the enzyme-bound state, the bisphosphonate of N-BPs is fully ionized to the *tetra* anion and interacts with three Mg^{2+} ions. These metal-mediated interactions allow binding of N-BPs to two highly conserved aspartate-rich (DDXXD) motifs that define the allylic sub-pocket (DMAPP/GPP-binding site) of the hFPPS active site. Until very recently, all known N-BPs were found to bind exclusively in this manner and only in the allylic sub-pocket of the enzyme. In a recent report, we identified for the first time thienopyrimidine-based bisphosphonates (e.g., **6**) that exhibit a mixed binding mode.[10] We provided DSF, NMR and crystallographic data which strongly suggested that inhibitors of general structure **6** can bind mainly in the allylic sub-pocket of hFPPS in the presence of Mg^{2+} ions and in the allosteric pocket of hFPPS in the absence of Mg^{2+} ions (PDB IDs: 4JVJ vs 4LPG, respectively). The relative contributions of the two binding modes under biologically relevant conditions were estimated by ITC.[10] However, since in vitro evaluation of hFPPS activity requires Mg^{2+} as the cofactor (thus biasing the binding of bisphosphonates to the allylic sub-pocket), the ability of bisphosphonates to bind in part or exclusively in the allosteric pocket under biologically relevant conditions remained difficult to prove. In this report, we describe the design and synthesis of novel bisphosphonate inhibitors of hFPPS that are structurally related to the clinical drug **5**. We provide data which strongly suggests that some of these inhibitors can occupy simultaneously both the allylic sub-pocket and IPP sub-pocket of the active site cavity. In addition, we obtained conclusive evidence that bisphosphonates inhibitors of hFPPS can occupy the allosteric pocket of the enzyme, even in the presence of high concentrations of Mg^{2+} ions. These findings, together with the fact that bisphosphonates are structural mimics of isoprenyl metabolites (i.e., mimics of DMAPP and GPP), support the previously proposed theory that the allosteric pocket of hFPPS plays a regulatory role in product feedback inhibition.

Based on the previously reported binary and ternary structures of hFPPS/N-BP and hFPPS/N-BP/IPP complexes, it is well established that the initial occupancy of the allylic sub-pocket by an N-BP inhibitor leads to a structural change of the active site cavity from the fully 'open' to the 'half-closed' conformation.[3] This conformational transition of the protein re-defines the shape of the IPP binding sub-pocket, allowing high-affinity binding of the homoallylic substrate (IPP). Co-occupancy of the allylic sub-pocket and the IPP sub-pocket sets in motion a second conformational change, which involves folding of the C-terminal residues ^{350}KRRK^{353} over the IPP sub-pocket and complete 'closing' of the active site cavity, thus sequestering both bound ligands from bulk water. In contrast, binding of an allosteric inhibitor near the IPP sub-pocket freezes the active site cavity in the catalytically incompetent open (or half closed) conformation, even in the presence of a co-bound N-BP (PDB code: 3N46) and blocks binding of IPP; more specifically, it blocks the binding of the pyrophosphate moiety of IPP. Based on all these data, the role of the lipophilic tail of IPP in the binding contributions and the biological relevance of the allosteric pocket remained unclear.

We noted that in the enzyme bound state, the side chains of N-BP inhibitors are within van der Waals radius from the IPP hydrophobic tail (Fig. 1a–c). We also recently showed that bisphosphonate **6b** binds mainly in the allylic sub-pocket, but its pyrimidine ring protrudes into the IPP sub-pocket, thus occupying part of the region that is normally occupied by the IPP isoprenyl tail (PDB code: 4L2X; Fig. 1c). Consequently, we reasoned that N-BPs with an appropriate molecular design could possibly occupy both the allylic and IPP sub-pockets simultaneously and potentially exhibit much higher affinity for the active site cavity. Structural characterization of the interactions between such inhibitors and the enzyme could lead to the designs of novel hFPPS inhibitors with lower dependency on the bisphosphonate anchor for binding to the active site cavity.

Initially, we used an in silico model to dock benzimidazole bisphosphonates of general structure **8c** to the fully closed active site of hFPPS using GLIDE (version 5.5, Shrödinger, LLC, New York, NY 2009; standard parameters of XP-mode were used). Favourable outputs for the binding of the R^1 alkyl moiety into the IPP sub-pocket were obtained; an example of a docked molecule, where the R^1 substituent of **8c** is $-C(CH_3)$cyclopropyl, is shown in Figure 1d. However, mindful of the significant protein plasticity of hFPPS and the large conformational changes previously observed upon ligand binding to this target, we were cautious about the validity of these data.

Benzimidazole-based derivatives (e.g., **8a**,**b**) have been previously reported as potent inhibitors of hFPPS.[11] We initiated the synthesis of analogs with general structure **8c**, assuming that the R^1 alkyl group