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The inhibition of human farnesyl pyrophosphate synthase by nitrogen-containing bisphosphonates. Elucidating the role of active site threonine 201 and tyrosine 204 residues using enzyme mutants☆

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ABSTRACT

Farnesyl pyrophosphate synthase (FPPS) is the major molecular target of nitrogen-containing bisphosphonates (N-BPs), used clinically as bone resorption inhibitors. We investigated the role of threonine 201 (Thr201) and tyrosine 204 (Tyr204) residues in substrate binding, catalysis and inhibition by N-BPs, employing kinetic and crystallographic studies of mutated FPPS proteins.

Mutants of Thr201 illustrated the importance of the methyl group in aiding the formation of the Isopentenyl pyrophosphate (IPP) binding site, while Tyr204 mutations revealed the 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), although RIS was also still capable of interacting 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.

In conclusion we have identified new features of FPPS inhibition by N-BPs and revealed unknown roles of the active site residues in catalysis and substrate binding.

1. Introduction

Farnesyl pyrophosphate synthase (FPPS) is a key branch point enzyme in the mevalonate pathway, the exclusive route of isoprenoid production in animals, involved in cholesterol biosynthesis and synthesis of intermediates important for intracellular signalling and growth control [1]. Inhibition of FPPS causes a reduction in farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) levels leading to impaired prenylation and defective intracellular localization of GTPase signalling proteins such as Ras, Rho and Rac [2,3]. These proteins are essential for osteoclast cell processes such as bone resorption, cell movement, cytoskeletal rearrangement and apoptosis [4,5]. FPPS has been confirmed as the primary molecular target of the nitrogen-containing bisphosphonates (N-BPs) that are potent inhibitors of osteoclastic activity [6]. Several members of this class are important clinically

Abbreviations: DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; N-BP, nitrogen-containing bisphosphonates; TCEP, tris(2-carboxyethyl)phosphine; RIS, Risedronate; ZOL, Zoledronate; PAM, Pamidronate; ALN, Alendronate; IBN, Ibandronate.

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used drugs for conditions characterized by excessive bone resorption such as Paget's disease [7], multiple myeloma [8], bone metastases [9] and osteoporosis [10,11].

FPPS is a homodimeric enzyme, made up of two 42 kDa monomers, that initially catalyse a head-to-tail condensation of the 5-carbon allylic substrate dimethylallyl pyrophosphate (DMAPP) with isopentenyl pyrophosphate (IPP) to yield the C_{10} geranylpyrophosphate (GPP). Subsequently, a second head-to-tail condensation of GPP and IPP within this enzyme yields the C_{15} farnesyl pyrophosphate (FPP). The reaction is thought to proceed by a highly ordered three step ionization-condensation-elimination mechanism, via the formation of a carbocation intermediate, which is stabilized by the presence of the OH group of Thr201, the main-chain carbonyl oxygen of Lys200 and the side chain oxygen of glutamine 240 [12–14]. The ionization of DMAPP is facilitated by the enzyme-bound tri-nuclear Mg^{2+} cluster where all three Mg^{2+} ions form salt-bridges with the un-esterified oxygens of the pyrophosphate, enhancing the juxtaposition of the hydrophobic C_5 isoprenoid tail of the IPP into the correct conformation for subsequent catalysis [12,15]. The carbocation formed condenses with the nucleophilic C_3–C_5 double bond of IPP. Finally, elimination of the isoprenoid reaction product, GPP, is achieved by de-protonation of the condensed intermediate by the free pyrophosphate oxygen [12].

The structures of human FPPS co-crystallised with several clinically utilised N-BPs demonstrated that N-BPs occupy the "allylic substrate binding pocket" (DMAPP/GPP) of FPPS, in agreement with the proposed kinetic model [16,17]. Coordination of the phosphonate groups of N-BP is facilitated via interactions of Mg^{2+} ions with the same aspartate-rich motifs of FPPS that bind the pyrophosphate moieties of the allylic substrate [16]. In the case of two of the most potent inhibitors of FPPS, risedronate (RIS) and zoledronate (ZOL), the N-BP binding is strengthened by hydrogen bond interactions of the protonated nitrogen atom within the heterocyclic ring of the side chain of the N-BP with the conserved main-chain carbonyl oxygen of Lys200 and the hydroxyl group of the Thr201 side chain, mimicking the stabilization of a carbocation intermediate [12,16–18]. In the case of pamidronate (PAM), the position of the hydroxyl of the Tyr204 is found close to the amino-group in the alkyl side chain of PAM (3 Å) and, favours formation of a hydrogen bond thus accounting for the intermediate to weak enzyme inhibitory effect of PAM [17]. The N-BP:FPPS interactions are complex in nature due to N-BPs having two different modes of inhibition: a rapid and reversible competitive inhibition with regard to the allylic substrate DMAPP/GPP, reflecting the inhibition constant Ki, and an uncompetitive or mixed-type inhibition in relation to IPP [16,19]. The latter is a time-dependent inhibition, arising due to an enzyme isomerization occurring in two stages: i) N-BP binds to the allylic site and forms the IPP binding site and ii) IPP binds to the FPPS:N-BP complex and closes the active site using amino acid residues found in the C-terminus of FPPS (Lys350, Arg351, Arg352, Lys353) [16,17]. This complex inhibition mechanism is expressed as a final isomerized inhibition constant Ki*. The tendency of the enzyme to remain in the isomerized state is given by the isomerization constant, $K_{isom} = (Ki - Ki^*) / Ki^*$.

The biological effects of the bisphosphonates are still being extensively studied and many new applications are emerging, including improvements to current therapies [20,21]. There is also interest in mevalonate pathway modulation as a potential target for the treatment of diseases caused by protozoan parasites, such as toxoplasmosis [22], leishmaniasis [23,24], Chagas disease [25,26] and malaria [27,28]. An understanding of the molecular interaction of pivotal FPPS amino-acid residues with the side chain of selected BPs (Table 1) may enable the synthesis of additional N-BP analogues that will selectively target the FPPS enzymes in humans and other species. For the applications focused more on non-skeletal tissue targets, developments in the design and measurements of lower bone affinity bisphosphonates have evolved that may advance the utility of the class, both with regards to lower skeletal uptake and enhanced activity [6].

Table 1
BPs used in the study.