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Synthesis, modelling and kinetic assays of potent inhibitors of purple acid phosphatase

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ABSTRACT

Purple acid phosphatases (PAPs) are binuclear metallohydrolases that have been isolated from various mammals, plants, fungi and bacteria. In mammals PAP activity is associated with bone resorption and can lead to bone metabolic disorders such as osteoporosis; thus human PAP is an attractive target to develop anti-osteoporotic drugs. Based on a previous lead compound and rational drug design, acyl derivatives of α-aminonaphthylmethylphosphonic acid were synthesised and tested as PAP inhibitors. Kinetic analysis showed that they are good PAP inhibitors whose potencies improve with increasing acyl chain length. Maximum potency is reached when the number of carbons in the acyl chain is between 12 and 14. The most potent inhibitor of red kidney bean PAP is the dodecyl-derivative with K_{ic} = 5 μM, while the most potent pig PAP inhibitor is the tetradecyl-derivative with K_{ic} = 8 μM, the most potent inhibitor of a mammalian PAP yet reported.

Purple acid phosphatases (PAPs)[1] are binuclear metallohydrolases which belong to the superfamily protein phosphatases (PPs)[2–4] and have a characteristic purple colour due to a metal–ligand charge transfer from a tyrosine phenolate to a chromophoric Fe(III) in the active site.[5] They utilise heterovalent metal ions (Fe(III)M(II); M = Fe (mammals), Zn or Mn (plants))[5–8] in their active sites to bring about phosphoric ester hydrolysis,[1,6,9] especially at neutral to acidic pH[5,10,11] according to the equation:[12]

$$RO\text{-}PO_3^{2-} + H_2O \rightarrow ROH + HPO_4^{2-} \quad (1)$$

Mammalian PAPs (also known as tartrate-resistant acid phosphatase (TRAP)[13] or osteoclastic acid phosphatase (OAP)[14]) are ~35 kDa monomeric proteins, while plant PAPs are homodimers with molecular weight of approximately 100–120 kDa, with each subunit connected through a disulfide bridge.[1,15,16] While the sequence homology between animal and plant PAPs is low, the amino acids in the active site, and the spatial arrangements of the active site residues, are highly conserved.[17] For these reasons, all PAPs are believed to use similar mechanistic strategies.[1,5,7,11,15]

The biological roles of PAPs are diverse and not yet fully explored.[12] The association of mammalian PAP with osteoporosis[15,18–20] has prompted us to examine its potential as a therapeutic target for the treatment of this disease, and to develop potent inhibitors of this enzyme.[12,21] While clinical treatments are available for osteoporosis, notably bisphophonates which inhibit farnesyl pyrophosphate synthase in osteoclasts,[22,23] these drugs have significant side effects and compliance issues. Mammalian PAP is secreted into osteoclasts, the giant multinucleated bone resorbing cells,[24,25] where it plays a role in bone turnover by increasing bone resorption.[1,13,15,26–30] Increased bone resorption that exceeds bone formation creates an imbalance in the dynamic bone remodelling process,[31] which is the major factor in osteoporosis development. Evidence of PAP's role in bone resorption includes the development of osteopetrosis (the opposite phenotype of osteoporosis) in PAP knockout mice,[32] and that over-expression of PAP in transgenic mice results in them becoming osteoporotic.[33] Furthermore, osteoporosis patients have elevated PAP serum levels,[15,34] and reducing PAP activity using a PAP-specific antibody has been shown to hinder osteoclastic bone resorption in an in vitro bone resorptive assay.[26,27] These reports therefore identify human PAP (hPAP) as a very important target for the development of anti-osteoporotic drugs.

Fluoride[10,35–37] and a number of simple tetrahedral inorganic oxyanions, such as phosphate, arsenate, vanadates,[38] tungstate and molybdate[26,39] are weak and non-specific inhibitors of PAP. Simple phosphonate-containing molecules with pendant metal-binding groups such as carboxylate, thiol and phosphonate have been shown to inhibit red kidney bean PAP (rkbPAP) with IC_{50} values of 80–3000 μM,[40] and several modified phosphotyrosine-containing tripeptides have also shown inhibitory activity towards several mammalian and plant PAPs, with IC_{50} values in the mid-micromolar range.[34] Recently our group reported the potent inhibitory activities of a series of α-alkoxynaphthylmethylphosphonic acids, **1**, with K_i and IC_{50} values against pig PAP (pPAP)

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and rkbPAP in the low micromolar range.[12] These compounds were designed as derivatives of a 1-naphthylmethylphosphonic acid template, **2**, previously reported by Schwender et al.[14] The long alkyl chain moieties of these compounds were incorporated with the expectation that they could bind favourably to a prominent channel on the surface of PAP adjacent to the active site of the enzyme. This supposition was supported by molecular modelling and the finding that, in general, longer alkyl chains in **1** led to inhibitors with higher potencies.[14]

1 **2**

R = hexyl, octyl, decyl

Here, we report the design, synthesis and inhibitory activities of a second-generation series of potent PAP inhibitors, **6**. The key structural feature of this new class of inhibitor is the substitution of ether linkage in compound **1** with a secondary amide bond. This functional group was introduced for several reasons: (i) the amide group was expected to improve the water solubility of these compounds; (ii) Schwender had previously reported that PAP inhibitors bearing an acyl group proximal to the phosphonate moiety were superior to those that did not,[14] suggesting favourable hydrogen-bonding interactions between the carbonyl group of the inhibitors and a surface residue near the enzyme's active site, and (iii) these inhibitors were expected to be easier to synthesise than analogues containing ether linkages. Therefore inhibitors of general structure **6** with varying lengths of acyl chains were examined. In this study rkbPAP and pPAP were used to assay these inhibitors. Although human PAP (hPAP) would be the best choice for our purposes, hPAP can only be obtained in minute quantities using a baculoviral recombinant expression system.[41] However, rkbPAP and pPAP are valid models of hPAP, as indicated by the highly conserved active sites across these different enzymes,[1] the similarities in substrate-binding pockets,[11,42–46] and the similarities of inhibition constants for a range of structurally different inhibitors reported for several animal (including human) and plant PAPs.[1,10,12,34,35,46–48]

Computer modelling was used to determine potential binding interactions between the inhibitors **6a–g** and hPAP, rkbPAP and pPAP. Crystal structures of these three PAPs were downloaded from the Protein Data Bank (PDB). As **6a–g** were synthesised as racemates, docking simulations for both (*R*)- and (*S*)-isomers of each inhibitor were performed using *Molegro Virtual Docker* (MVD, Molegro ApS)[49] to determine whether enantiomers would have the same or different predicted binding affinities for the different PAP enzymes. The binding orientations of the inhibitors to the enzymes were predicted by MVD based on energy minimisation. The binding affinities of the compounds to the enzymes were represented by docking scores (MolDock Scores).[49] The docking scores for each pair of enantiomers docked on all three PAPs show that each isomer had similar predicted binding energies (see Table in Supplementary data), suggesting that each would have similar binding affinities for the enzymes. The docking scores also suggested a trend of inhibitors **6a–g** to have increased predicted binding affinity with increased chain length. However, it is observed that both (*R*)- and (*S*)-isomers of **6** reach maximal potency when R = $C_{13}H_{27}$–(**6f**) against pPAP, which was later confirmed by kinetic analysis (see below). On the other hand, variations in the docking scores were observed with increasing chain lengths between the two isomers on both hPAP and rkbPAP; where both hPAP and rkbPAP have the highest affinities for (*R*)-**6d** among the (*R*)-isomers, while (*S*)-**6e** and (*S*)-**6g** have the highest binding affinities for hPAP and rkbPAP, respectively, among the (*S*)-isomers.

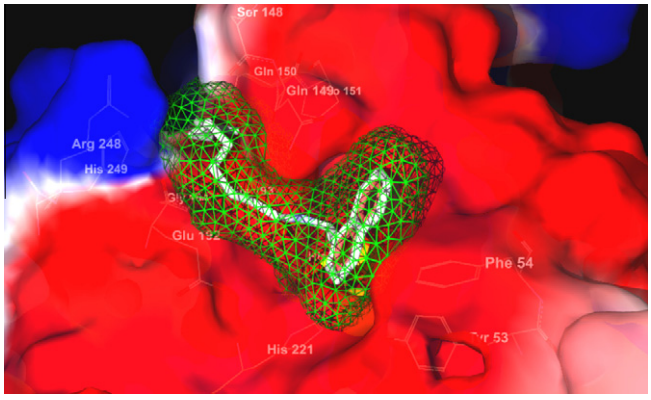
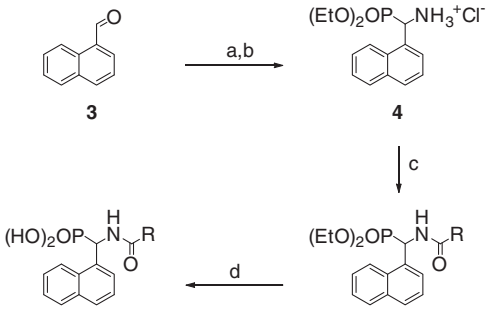


Figure 1. The predicted binding mode of (*R*)-**6f** in the active site of hPAP.

As expected, modelling suggests that the phosphonate moiety of inhibitors **6a–g** binds to the dimetal centre in the active site of all three enzymes, regardless of their stereochemistry. Furthermore, the alkyl chains of the (*R*)-isomers of the inhibitors bind to the groove on the surface of hPAP (Fig. 1), similar to the binding mode reported for **1**.[12] Interestingly, the alkyl chains of (*R*)-isomers of **6** do not bind to the groove in pPAP, instead they bind to the pocket, which is nearer to the dimetal centre than the surface groove (data not shown). This predicted binding orientation is consistent for all derivatives of (*R*)-**6** in the active site of pPAP. The similar binding orientations predicted for each isomer of the inhibitors **6a–g** in the active sites of both pPAP and hPAP support the contention that the inhibitory potencies of **6a–g** obtained from the kinetic studies using rkbPAP and pPAP can be extrapolated to hPAP.

The synthesis of inhibitors **6a–g** is shown in Scheme 1. All compounds were synthesised as racemates. The conversion of 1-naphthaldehyde **3** into diethyl (amino(naphthalen-1-yl)-methyl)phosphonate was achieved by heating **3** with a mixture of ammonium acetate and diethyl phosphite over activated molecular sieves in ethanol, according to a literature procedure reported for the synthesis of diethyl α-aminobenzylphosphonate from benzaldehyde.[50] Introducing hydrogen chloride gas to the free amine product gave its corresponding hydrochloride salt **4** in 23% overall yield.[50] Attempts to prepare **4** from **3** using the method described by Kaboudin and Moradi[51] were unsuccessful.

Acylations of **4** to give the amides **5a–g** were achieved without incident using appropriate acid chlorides, themselves prepared by



a, R = nC_3H_7 **b**, R = nC_6H_{13} **c**, R = nC_7H_{15}
d, R = nC_9H_{19} **e**, R = $nC_{11}H_{23}$ **f**, R = $nC_{13}H_{27}$
g, R = $nC_{15}H_{31}$

Scheme 1. Reagents and conditions: (a) diethyl phosphite, NH_4OAc, EtOH, 3 Å MS, 60 °C, 44 h; (b) HCl, EtOH–Et_2O, 23% (two steps); (c) RCOCl, DIPEA, CH_2Cl_2, rt, 18 h, 56% (**5a**), 77% (**5b**), 73% (**5c**), 71% (**5d**), 67% (**5e**), 67% (**5f**), 67% (**5g**); (d) TMSCl, NaI, MeCN, 40 °C, 18 h, 45% (**6a**), 38% (**6b**), 29% (**6c**), 68% (**6d**), 46% (**6e**), 23% (**6f**), 35% (**6g**).

Table 1
Kinetic data for inhibitors against pPAP and rkbPAP at pH 4.9

Inhibitor	pPAP		rkbPAP	
	K_{ic} (mM)	K_{iuc} (mM)	K_{ic} (mM)	K_{iuc} (mM)
6a	0.363 ± 0.165	1.439 ± 1.510	0.238 ± 0.010	0.654 ± 0.240
6b	0.116 ± 0.045	0.349 ± 0.225	0.222 ± 0.109	0.446 ± 0.144
6c	0.044 ± 0.013	––	0.195 ± 0.120	0.443 ± 0.198
6d	0.010 ± 0.003	––	0.057 ± 0.024	0.102 ± 0.026
6e	0.021 ± 0.007	––	0.005 ± 0.002	––
6f	0.008 ± 0.001	––	0.011 ± 0.004	––
6g	0.013 ± 0.003	––	0.031 ± 0.019	––

K_{ic}, Competitive inhibition constant.
K_{iuc}, Uncompetitive inhibition constant.
––, no significant uncompetitive inhibition.

refluxing the corresponding carboxylic acids with thionyl chloride.[52] The product amides were purified by flash column chromatography to give **5a–g** in good yields. The final synthetic step to produce the newly designed inhibitors **6a–g** required the cleavage of the phosphonate ester bonds of **5a–g** to give their corresponding free phosphonic acids. This was achieved by heating **5a–g** with a mixture of sodium iodide and trimethylsilyl chloride in acetonitrile at 40 °C[12] giving the desired products in moderate yields.

The inhibitory effects of phosphonates **6a**–**g** were tested against both pPAP and rkbPAP at pH 4.9.[34,35] The results are listed in Table 1. The shorter-chain inhibitors **6a**–**b** exhibited mixed mode inhibition (competitive and uncompetitive) for pPAP, and inhibitors **6a**–**d** also showed this behaviour against rkbPAP. Such mixed mode inhibition has also been reported for our previously reported inhibitors.[12] For longer chain-length inhibitors, only competitive inhibition was observed against both pPAP and rkbPAP. This behaviour may reflect a stronger anchoring effect of the longer alkyl chains into the groove adjacent to the active site of the enzyme, which would favour competitive inhibition. In agreement with our modelling studies, the inhibitory potencies of these compounds generally increased with increasing chain length, reaching a maximum K_{ic} of 8 μM for **6f** against pPAP and 5 μM for **6e** against rkbPAP.

In conclusion, new inhibitors of pPAP and rkbPAP have been identified using computer-aided design, and these have been shown to exhibit potent inhibitory activities against these enzymes. Compound **6e** (K_{ic}, 5 μM) is comparable to that of the most potent inhibitor of rkbPAP (4 μM)[12] and compound **6f** (K_{ic}, 8 μM) is the most potent inhibitor of pPAP yet described. In addition, it has also been shown that predicted binding affinities calculated using computational modelling correlate well with measured inhibition constants for this class of inhibitor.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.024.

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