



# Design and synthesis of selective inhibitors of Placental Alkaline Phosphatase

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## ABSTRACT

Placental Alkaline Phosphatase (PLAP) is a tissue-restricted isozyme of the Alkaline Phosphatase (AP) superfamily. PLAP is an oncodevelopmental enzyme expressed during pregnancy and in a variety of human cancers, but its biological function remains unknown. We report here a series of catechol compounds with great affinity for the PLAP isozyme and significant selectivity over other members of the AP superfamily. These selective PLAP inhibitors will provide small molecule probes for the study of the pathophysiological role of PLAP.

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## 1. Introduction

Alkaline phosphatases (APs) are found in many organisms from bacteria to humans.<sup>1,2</sup> APs are a subgroup of a superfamily of metalloenzymes with similar metal-binding sites and predicted conserved protein structural folds.<sup>3</sup> In humans, APs are encoded by four genes traditionally named after the tissues where they are predominantly expressed. The tissue-nonspecific AP (TNAP) gene is expressed at greatest levels in liver, bone, kidney, in the placenta during the 1st trimester of pregnancy, and at lower levels in numerous other tissues.<sup>1,2</sup> The other three isozymes, (i.e., placental AP (PLAP), germ cell AP (GCAP), and intestinal AP (IAP)), show a much more restricted tissue expression, generally referred to as tissue-specific APs. Tissue-specific PLAP, GCAP and IAP are 90–98% homologous while TNAP is only 50% identical with the other three APs. Most of what we know about function comes from studies of the biological roles of TNAP and more recently IAP. TNAP is essential for proper skeletal mineralization. Its role is to hydrolyze inorganic pyrophosphate, a potent calcification inhibitor, via its pyrophosphatase activity.<sup>4–7</sup> Lack of TNAP leads to infantile hypophosphatasia, a lethal form of rickets and osteomalacia in mice, rescued by enzyme replacement therapy.<sup>8</sup> IAP appears to have a role in fat absorption in the gastrointestinal tract<sup>9,10</sup> and as a gut mucosal defense factor<sup>11</sup> where its expression coincides with the stage of colonization by commensal bacterial flora.<sup>12,13</sup> In contrast, relatively little is known about the functions of PLAP

and GCAP largely due to the fact that these genes evolved only recently, having appeared during a late evolutionary duplication event that preceded the divergence of Old World Monkeys and Apes.<sup>1</sup>

PLAP is expressed in large amounts in the syncytiotrophoblast cells of the placenta from about the eighth week of gestation throughout pregnancy.<sup>14</sup> PLAP was one of the first enzymes recognized as an oncofetal protein expressed in a variety of cancers.<sup>15,16</sup> GCAP is also found expressed in the placenta although at levels 50-fold lower than PLAP<sup>17</sup> and it is re-expressed in testicular germ cell tumors of the testis, particularly seminomas.<sup>18,19</sup> PLAP and GCAP are often co-expressed in ovarian cancer<sup>20</sup> and it has been suggested that transformation of normal to malignant trophoblast might be associated with a switch from PLAP to GCAP expression.<sup>21</sup> Thus, the ability to discriminate between these AP isozymes might facilitate studies aimed at uncovering their pathophysiological function.

Several isozyme-selective inhibitors of APs have been reported (Fig. 1), including L-Phe, L-Trp, L-Leu<sup>22,23</sup>, L-homoarginine<sup>24</sup> and also some unrelated compounds, such as L-leucinamide, levamisole, the L-stereoisomer of tetramisole<sup>25</sup>, and theophylline (a 1,3-dimethyl derivative of xanthine).<sup>26</sup> Inhibition by these agents is of a rare uncompetitive type<sup>27</sup> and the IC<sub>50</sub> of these inhibitors are reported to be above 5 μM for all three APs and poorly selective.<sup>28,29</sup> Because of the great homology between PLAP, GCAP and IAP, no selective inhibitors of the PLAP enzymes have been reported until now. In this report, we describe the development of potent PLAP inhibitors that possess selectivity over TNAP and IAP and moderate selectivity over GCAP.

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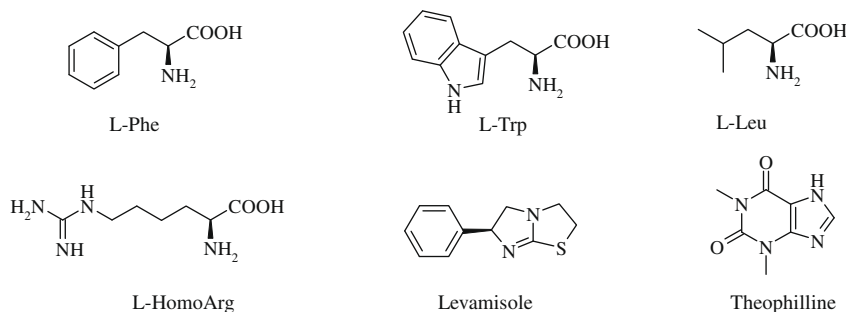


Figure 1. Examples of AP inhibitors.

A library of 95,857 compounds<sup>30</sup> was tested in a luminescent HTS assay<sup>31</sup> for discovery of chemical inhibitors of PLAP. Initially, 192 'hits' (i.e., 0.2%) were identified as having at least 50% inhibition of PLAP functional activity in an assay at 20  $\mu$ M. Eighty two of these hits were confirmed in dose-response experiments for inhibition of PLAP function.<sup>31</sup> In parallel studies, the 82 confirmed hits were also tested for inhibition selectivity against TNAP functional activity. A few confirmed hits contained a catechol moiety and were selected as a lead series. The catechol series was chemically stable and showed early trends of a structure activity relationship (SAR). Compound **1** (Fig. 2) was the most potent inhibitor 'hit' from the PLAP inhibition screening campaign. **1** possessed an  $IC_{50}$  of 2.5  $\mu$ M but was weakly selective over TNAP ( $IC_{50}$  = 8.6  $\mu$ M) and IAP ( $IC_{50}$  = 49.5  $\mu$ M) (Fig. 2).

A SAR was developed around **1** by varying all three regions (LHS, RHS and linker) of the catechol 'hit' **1** (Fig. 2) to obtain relatively selective PLAP inhibitors over the other members of the AP family.

## 2. Chemistry

For variation of the LHS and RHS regions of the original hit **1**, compounds **3–20** were readily synthesized using a one step synthesis by combining commercially available aryl substituted 2-chloroacetophenones **2** with various amines or thiols as shown in Scheme 1. Typically, the desired substituted 2-chloroacetophenone **2** was treated with 1 equiv of a nucleophile in dioxane either at room temperature or by heating the mixture up to 80 °C. Products **1**, **3–20** precipitated from the reaction mixture.

Exploration of the SAR of linker region of **1** (Fig. 2) necessitated using different starting materials. Compound **22** (Scheme 2) was prepared with standard reductive-amination conditions by treating a mixture of 3,4-dihydroxybenzaldehyde **21** and 2-amino-4,6-dimethylpyrimidine in the presence of triacetoxyborohydride in dichloroethane (DCE). Compounds **25** and **26** (Scheme 2) were obtained by treating 3,4-dihydroxybenzylamine **23** and 3,4-dihydroxyphenylethylamine **24**, respectively, with 4,5-dimethyl-2-furoic acid in the presence of standard peptide coupling conditions (HBTU, DIEA).

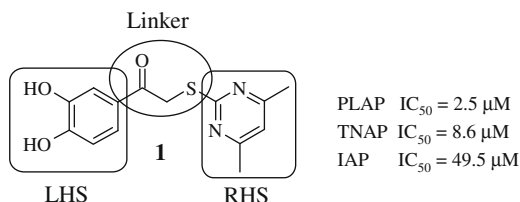
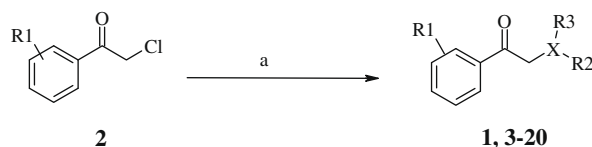
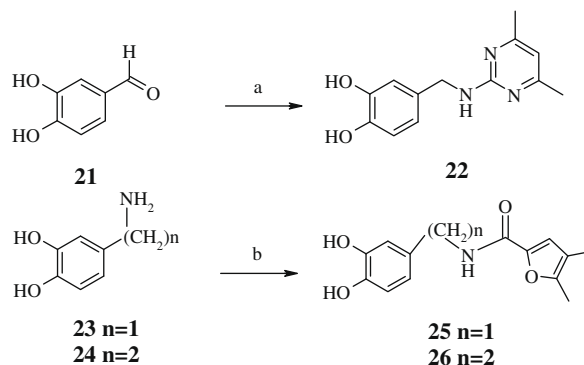


Figure 2. Depiction of the three regions of screening 'hit' **1** explored by chemical synthesis: Left Hand Side (LHS), Right Hand Side (RHS) and Linker region (encircled).



Scheme 1. Synthetic approach to explore the SAR of the LHS and RHS of screening 'hit' **1**; preparation of analogs **3–20**, X is nitrogen or sulfur. (a) 1 equiv HXR2R3, dioxane.



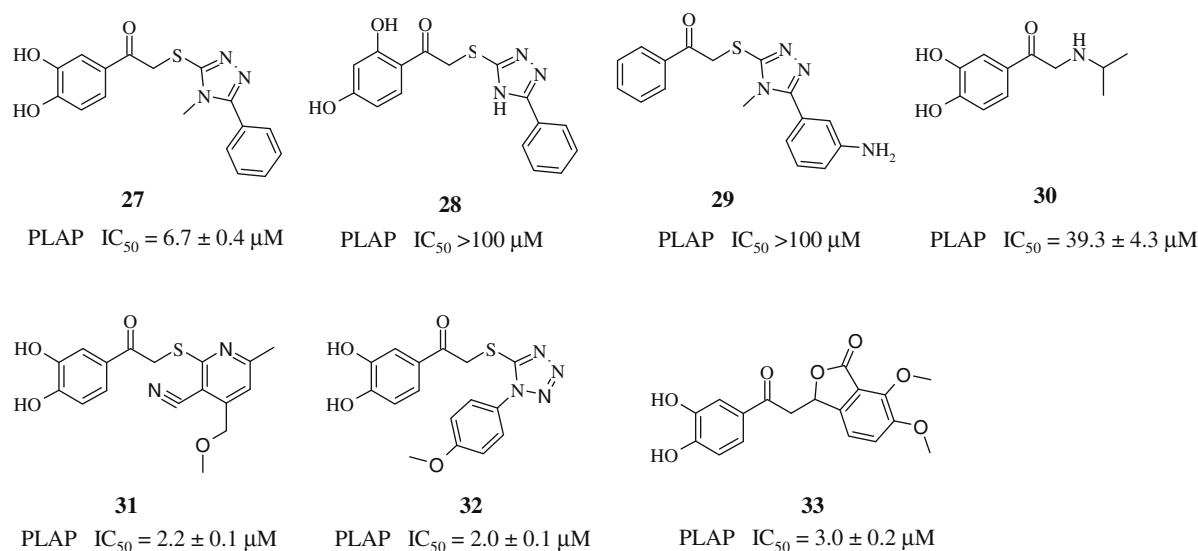
Scheme 2. Synthetic approach to explore the linker region of screening 'hit' **1**: preparation of analogs **22**, **25** and **26**. Reagents: (a) 2-amino 4,6-dimethyl pyrimidine, NaBH(OAc)<sub>3</sub>, DCE; (b) 4,5-dimethyl 2-furoic acid, HBTU, DIEA, DMF.

## 3. Results and discussion

To gain a quick understanding of the SAR requirements, analogs **27–33** (Fig. 3) were purchased and a sublibrary of 27 compounds (Tables 1–4) was synthesized according to the procedure illustrated in Schemes 1 and 2. Chemical re-synthesis of compound **1** (Table 1) was done according to Scheme 1 to compare an authentic sample with the commercially purchased compound in the bioassays used. Resynthesized **1** was found to have similar PLAP inhibitory potency as the library compound. Compounds **3–33** were compared to **1** in the enzyme inhibition assays (i.e., PLAP, TNAP) to characterize enzyme inhibition potency and selectivity. The most active compounds in the PLAP assay were also tested against IAP and GCAP.

Based on several purchased analogs (three representative examples, **27**, **31–33**, are shown in Fig. 3), it was rapidly recognized that the 3,4-dihydroxyl arrangement of the catechol in the LHS seemed optimal for inhibition of alkaline phosphatase. Compound **27** (Fig. 3) had an  $IC_{50}$  of 6.7  $\mu$ M whereas **28** (Fig. 3) and **29** (Fig. 3) that did not have a 3,4-dihydroxyl group were not potent ( $IC_{50}$  >100  $\mu$ M) PLAP inhibitors.

A more thorough exploration of the SAR of the LHS aryl substituent of compound **1** (Table 1) confirmed that the 3,4-dihydroxy



**Figure 3.** Examples of commercially available analogs **27–33** purchased and their  $IC_{50}$  values in the PLAP assay.

**Table 1**  
 $IC_{50}$  values for PLAP inhibition when the left hand side was varied

Compound number	<b>1</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
PLAP potency ( $IC_{50}$ ) $\mu M$ <sup>a</sup>	$2.5 \pm 0.1$	>100	>100	>100	>100	>100

<sup>a</sup> Values are the mean  $\pm$  SD of three experiments.

**Table 2**  
 $IC_{50}$  values for PLAP inhibition when the linker region of **1** was varied.

Compound number	<b>1</b>	<b>22</b>	<b>25</b>	<b>26</b>
PLAP activity(IC <sub>50</sub> ) μM <sup>a</sup>	2.5 ± 0.1	51.2 ± 5.7	>100	>100

<sup>a</sup> Values are the mean  $\pm$  SD of three experiments.

**Table 3**  
 $IC_{50}$  values for PLAP and TNAP inhibition: Variation of the right hand side of the lead

Compound number	<b>1</b>	<b>30</b>	<b>31</b>	<b>32</b>	<b>33</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>
PLAP inh. potency <sup>a</sup> ( $IC_{50}$ ) $\mu M$	$2.5 \pm 0.1$	$39.3 \pm 4.3$	$2.2 \pm 0.1$	$2.0 \pm 0.1$	$3.0 \pm 0.2$	$2.1 \pm 0.2$	$13.4 \pm 1.1$	$2.7 \pm 0.1$	$9.1 \pm 0.6$
TNAP inh. potency <sup>a</sup> ( $IC_{50}$ ) $\mu M$	$8.6 \pm 0.5$	>100	$4.8 \pm 0.2$	$8.0 \pm 0.6$	$29.2 \pm 1.4$	>100	>100	>100	$93.8 \pm 16.2$

<sup>a</sup> Values are the mean  $\pm$  SD of three experiments.

phenyl substituent was crucial for inhibition of PLAP functional activity. Compared to lead compound **1**, removing one of the hydroxyl groups (i.e., **3**, **4**), capping one (i.e., **5**, **7**), or capping both hydroxyl groups (i.e., **6**) afforded compounds with no PLAP inhibitory potency at 100  $\mu M$  (Table 1).

A brief SAR survey of the linker region was done by synthesis and it was observed that synthetic modification of the linker region of **1** led to a significant decrease of potency of PLAP inhibition. Thus, shortening the linker by one atom spacer and removing the ketone moiety afforded a 25-fold decrease in potency of inhibition

**Table 4**  
IC<sub>50</sub> values for inhibition of PLAP, TNAP and IAP: SAR of the right hand side of lead structure **1**

Compound number	<b>8</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>	<b>16</b>	<b>17</b>	<b>18</b>	<b>19</b>	<b>20</b>
PLAP potency <sup>a</sup> IC <sub>50</sub> μM	2.1 ± 0.2	3.3 ± 0.2	4.2 ± 0.3	17.2 ± 1.3	5.8 ± 0.2	15.8 ± 1	0.8 ± 0.05	5.8 ± 0.6	4.9 ± 0.6	1.2 ± 0.1
TNAP (IC <sub>50</sub> ) μM Selectivity <sup>a</sup> (TNAP/PLAP)	>100 (>32)	78.3 ± 12.9 (25)	>100 (>27)	>100 (>6)	41.1 ± 4.1 (7)	47.5 ± 5.5 (3)	18.0 ± 1.7 (9)	>100 (>17)	8.7 ± 0.7 (1.7)	26.3 ± 2.8 (8)

<sup>a</sup> Values are the mean ± SD of three experiments.

(e.g., compound **22**, Table 2). Compounds **25** and **26** (Table 2) that had a 4,5-dimethylfuran in place of the 4,6-dimethylpyrimidine as well as a different linker resulted in significant loss of inhibition potency.

Structural variation of the RHS side chain provided SAR information for further refinement of lead molecule **1**. Compound **30** (Table 3) and other RHS alkyl-substituted side-chains (not shown) suggested that an aromatic ring of the RHS was necessary for potent PLAP inhibition. The isopropyl amine analog **30** was the most potent of the alkyl-substituted side-chains with an IC<sub>50</sub> of 39.3 μM for PLAP inhibition (Table 3). Additional SAR studies with compounds maintaining a catechol group in the LHS showed that compounds tolerated a large number of RHS heteroaromatic rings (i.e., substituted thiopyridine **31**, substituted tetrazole **32**, benzofuranone **33**, imidazole **8**, triazole **9**, pyrazole **10**, 2-amino benzimidazole **11**, Table 3) and linkers (i.e., sulfur **1**, **31**, **32**; carbon **33**; nitrogen **30**, **8–11**, Table 3) in place of the pyrimidine moiety. However, such heteroaromatic compounds were also generally poor selective inhibitors of PLAP compared to TNAP (i.e., 2-, 4-, and 10-fold selectivity IC<sub>50</sub> PLAP/IC<sub>50</sub> TNAP for **31**, **32**, **33** and **11**, respectively, Table 3).

Exploration of the SAR of the RHS region of compound **1** with heterocycles such as imidazole, triazole or pyrazole directly attached to the core structure led to moderately potent PLAP inhibitors (IC<sub>50</sub> <14 μM) that possessed PLAP inhibition selectivity over TNAP (IC<sub>50</sub> >100 μM) (i.e., compounds **8–10**, Table 3). Encouraged by the large inhibition selectivity of **8–10** for inhibition of PLAP compared to TNAP, a more thorough examination of the SAR of the RHS was explored using substituted imidazoles. Keeping the catechol moiety (LHS, Fig. 2) of compound **1** unchanged, a library of substituted imidazoles was prepared and tested as inhibitors of all three isozymes (i.e., PLAP, TNAP and IAP) for inhibition potency and selectivity. Results are summarized in Table 4. The presence of a hydrogen (**8**) or a small alkyl group (i.e., methyl, **12** or ethyl, **13**) at the 2-position of the imidazole had little influence on PLAP inhibitory potency. All three compounds, **8**, **12** and **13**, had IC<sub>50</sub> values of approximately 3 μM. With the introduction of a bulkier group at the 2-position (i.e., phenyl, **14**), potency decreased fivefold (IC<sub>50</sub> of **14** = 17.2 μM). Substitution at the 4 position of the imidazole ring with a small group such as methyl (e.g., **15**, Table 4), maintained similar inhibitory potency as the unsubstituted imidazole, **8**. However, introduction of a larger group such as a bromine atom into the 4 position of the imidazole (i.e., **16**, Table 4) led to a decrease in potency by about sevenfold (i.e., IC<sub>50</sub> = 15.8 μM). Addition of a methyl group at the 2 position of compound **16** (i.e., compound **19**, Table 4) increased inhibitory potency threefold. Compound **19** had an IC<sub>50</sub> value of 5.0 μM compared to an IC<sub>50</sub> of 15.8 μM for **16**. The 2-benzimidazole analog (i.e., **17**) was one of the most potent inhibitors with an IC<sub>50</sub> of 0.8 μM and this result suggested that aromatic pi–pi interaction with the enzyme may be important. Extending the steric bulk of compound

**17** by introduction of an alkyl group (i.e., 5,6-dimethylbenzimidazole) to afford **18** decreased the potency by about threefold (i.e., IC<sub>50</sub> of **18** = 5.8 μM). Addition of a methyl at the 2 position of the benzimidazole of **17** to afford **20** maintained similar potency of inhibition compared with **17**.

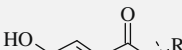








Within the imidazole series (Table 4), substitution at the 2-position did not seem to have a large effect on TNAP potency as the TNAP IC<sub>50</sub> for compounds **8**, **12–14** was greater than 78 μM leading to selectivity for PLAP inhibition over TNAP over 25-fold for an unsubstituted imidazole (**8**) or imidazoles bearing a small alkyl group such as methyl (**12**) or ethyl (**13**). Steric bulk at the 4- and or 5-position of the imidazole with compounds such as **15**, **16** or **19** and the benzimidazoles **17** and **20** showed a beneficial effect on the compound's ability to inhibit TNAP. The IC<sub>50</sub> for **15–17**, **19** and **20** were 41.1, 47.5, 18, 8.7 and 26.3 μM, respectively, with bromine being the optimum group size for the compounds tested (IC<sub>50</sub> for **19** = 8.7 μM). A larger group such as the 5,6-dimethylbenzimidazole (**18**) was not tolerated by the TNAP active site probably due to steric factors. The IC<sub>50</sub> for **18** was above 100 μM.

In summary, of the compounds examined (Table 4), the unsubstituted imidazole **8** was a considerably more selective inhibitor of PLAP compared with TNAP and had an IC<sub>50</sub> TNAP/IC<sub>50</sub> PLAP of over 32-fold.

The most potent inhibitors of PLAP (i.e., **1**, **8**, **11–13**, **17** and **20**, Table 5) were also tested as inhibitors of IAP and GCAP, the two other isozymes of the Alkaline Phosphatase family. Results are summarized in Table 5. Substitution at the 2 position of the imidazole appeared to be critical for selectivity of IAP inhibition. When the 2-substituent was changed from a hydrogen to a methyl to an ethyl group, the IC<sub>50</sub> for IAP inhibition increased, leading to increasingly less potent compounds (IC<sub>50</sub> = 30.2 μM, 64.6 μM, >100 μM respectively, for **8**, **12**, **13**). While the number of compounds examined was limited, a similar trend was also observed for the benzimidazole analogs **17** and **20**. Compound **17** had an IC<sub>50</sub> of 14.8 μM in the IAP assay while its 2-methylated version (i.e., compound **20**), did not show any detectable inhibition in the IAP assay (IC<sub>50</sub> >100 μM) that lead to a selectivity of over 80-fold for IAP over PLAP. Compounds shown in Table 5 were also tested for their inhibitory potential against GCAP. All of them were moderately potent inhibitors of GCAP with IC<sub>50</sub> values <10 μM. The IC<sub>50</sub> value for **1**, **8**, **11** and **17** was 0.6 μM, 1.2 μM, 6.2 μM and 2.7 μM, respectively. Also, compared to the inhibitory potency of compounds **1**, **8**, **11** and **17** on PLAP and GCAP (Fig. 4), compounds **1**, **8** and **11** can partially discriminate between PLAP and GCAP; the discrimination being optimal in the range around 1 μM while compound **12**, **13**, **17** and **20** had the same inhibitory potency for both isozymes at all inhibitor concentrations tested (graphs not shown for **12**, **13** and **20**).

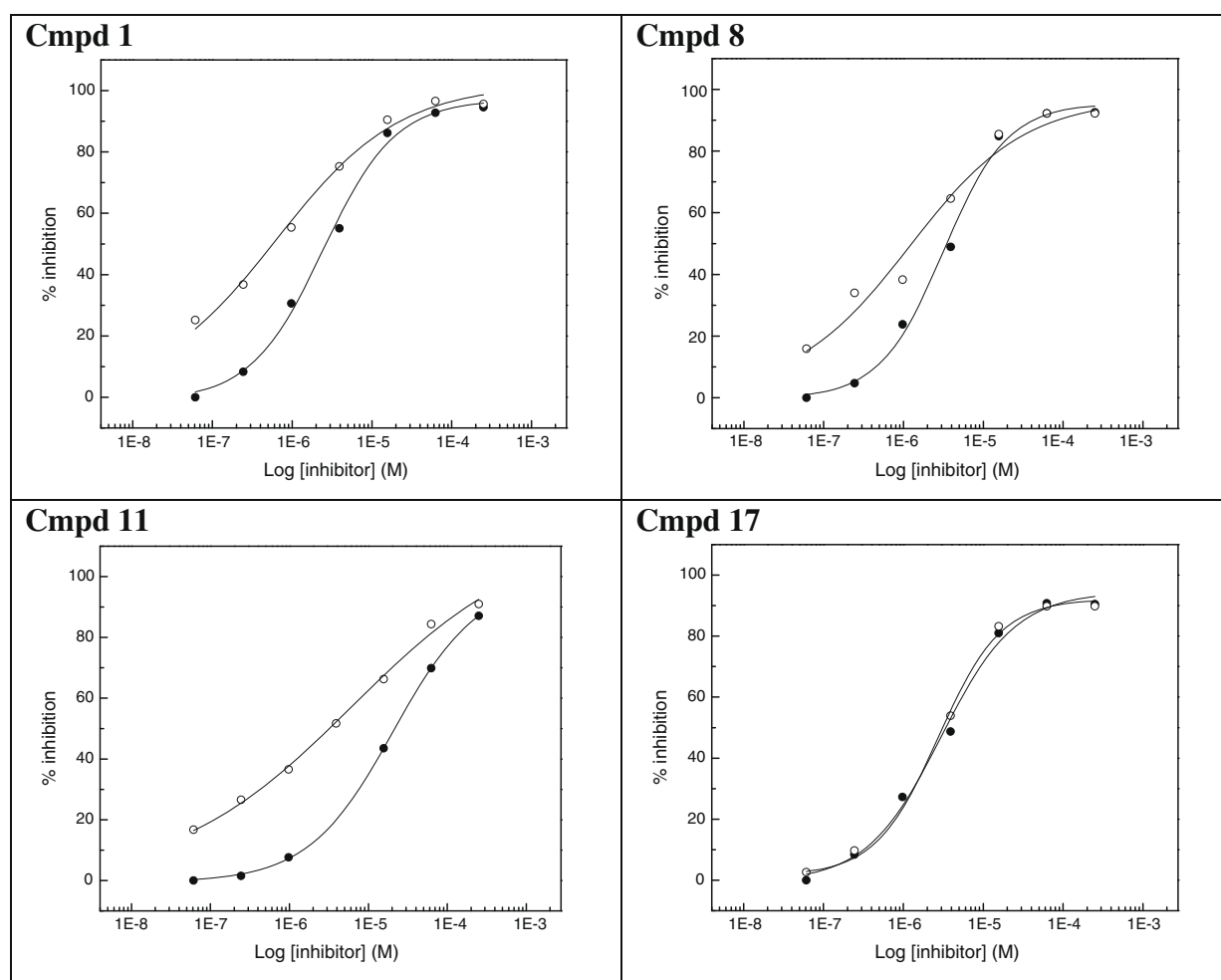
In summary, a series of 3,4-dihydroxy substituted catechols was developed that maintained inhibitory potency of the original screening hit **1** as an inhibitor of PLAP. Molecular modification of

**Table 5**  
Inhibitory activity for IAP and GCAP enzymes for compounds **1**, **8**, **10–13**, **17** and **20**

								
Compound number	<b>1</b>	<b>11</b>	<b>8</b>	<b>12</b>	<b>13</b>	<b>17</b>	<b>20</b>	<b>10</b>
PLAP potency IC <sub>50</sub> μM <sup>a</sup>	2.5 ± 0.1	9.1 ± 0.6	2.1 ± 0.2	3.3 ± 0.2	4.2 ± 0.3	0.8 ± 0.05	1.2 ± 0.1	2.7 ± 0.1
IAP (IC <sub>50</sub> ) μM <sup>a</sup>	49.5 ± 6.1	Nd	30.2 ± 2.5	64.6 ± 7.5	>100	14.8 ± 6.8	>100	53.3 ± 3.1
Selectivity (IAP/PLAP)	(20)		(15)	(20)	(>24)	(18)	(>80)	(25)
GCAP (IC <sub>50</sub> ) μM <sup>a</sup>	0.6	6.2	1.2			2.7		Nd
Selectivity (GCAP/PLAP)	(0.2)	(0.7)	(0.6)	(2.1)	(2.1)	(3.4)	(1.4)	

<sup>a</sup> Values are the mean ± SD of three experiments.

\* Compounds **12**, **13** and **20** were tested for GCAP and compared to PLAP on a different occasion. The enzyme concentrations were different. IC<sub>50</sub> values for PLAP and GCAP were 14.3, 30.5 μM for **12**; 0.8, 1.7 μM for **13** and 6.6, 9.4 μM for **20**, respectively.



**Figure 4.** Effect of increasing concentrations of compound **1**, **8**, **11** and **17** on the inhibition of PLAP (●) and GCAP (○).

**1** provided PLAP inhibitors with greater inhibition selectivity over two other related isozymes, TNAP and IAP (Tables 4 and 5). Compound **13**, possessing a 2-ethylimidazole substituent, was over 27-fold more selective as an inhibitor of PLAP compared to TNAP and IAP and maintained reasonable PLAP inhibitory potency (IC<sub>50</sub> = 4.2 μM). Compound **10** was more than 50- and 25-fold selective as an inhibitor of PLAP over TNAP and IAP, respectively (Fig. 5). Finally, compound **20** was 10- and 40-fold more selective an inhibitor of PLAP over IAP and TNAP, respectively. All three

compounds (i.e., **10**, **13** and **20**) were more potent inhibitors of PLAP than previously reported isozyme-selective inhibitors of APs (Fig. 1) and considerably more selective. We were also able to improve upon the selectivity of the screening hit **1**, especially with regard to TNAP selectivity. Compounds **1**, **8** and **11** are also somewhat selective for PLAP over GCAP in the one micromolar range. Because of their inhibitory selectivity, these catechol compounds may be useful as tools to understand the physiological role of PLAP in biology and pharmacology and may have applications



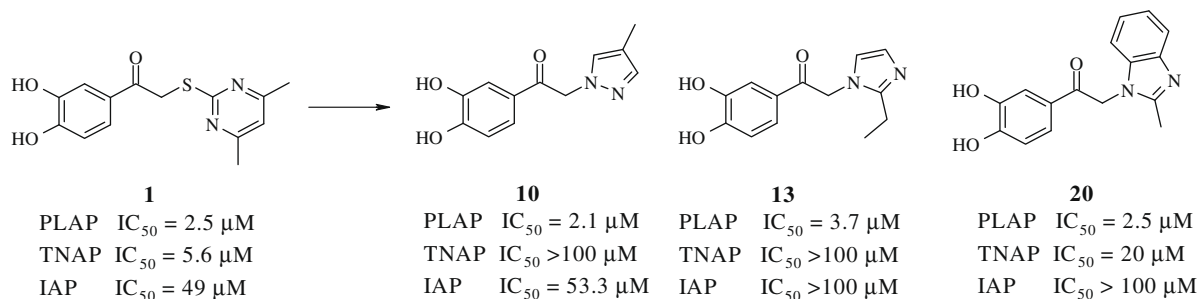


Figure 5. Summary of the  $IC_{50}$  values for selective PLAP inhibitors **10**, **13** and **20**.

for treating cancer or in cancer diagnostics because elevated levels of TNAP are found in ovarian and testicular cancer patients.

## 4. Experimental section

### 4.1. General methods and materials

Reagents, starting materials, and solvents were purchased from commercial suppliers in the highest quality available and were used as received. To concentrate refers to evaporation under vacuum using a Büchi rotary evaporator. Reaction products were purified, when necessary, by chromatography on silica gel (40–63  $\mu m$ ) with the solvent system indicated. Proton nuclear magnetic resonance data were recorded on a Varian Mercury 300 MHz Spectrometer using TMS as the internal standard. Carbon nuclear magnetic resonance data were recorded on a Bruker 300 MHz Spectrometer using TMS as the internal standard. Compounds were assessed for purity by HPLC–MS and were at least 85% pure at two wavelengths (224 nm and 254 nm). All tested compounds were characterized by  $^1H$  NMR and purity assessed by LC/MS-1 (Platform: Agilent: equipped with an auto-sampler, an UV detector (220 nm and 254 nm), a MS detector (APCI); HPLC column: Waters XTerraMS C18,  $3.0 \times 250$  mm; HPLC gradient: 1.0 mL/min, from 10% acetonitrile in water to 90% acetonitrile in water in 46 min maintaining 90% for 7.0 min. Both acetonitrile and water have 0.025% TFA.) or LC/MS-2 (Platform: Agilent 1100 series: equipped with an auto-sampler, an UV detector (230 nm and 254 nm), a MS detector (APCI); HPLC column: Phenomenex Synergi-Max RP,  $2.0 \times 50$  mm column; HPLC gradient: Solvent C is 6 mM Ammonium Formate in water, solvent D is 25% acetonitrile in methanol. The gradient runs from 5% D (95% C) to 95% D (5% C) in 6.43 min with a 1.02 min hold at 95% D followed by a return and hold at 5% D for 1.52 min) ( $t_R$  is retention time (TIC% purity).

### 4.2. TNAP, PLAP and IAP Luminescent assays<sup>31</sup>

Briefly, the TNAP assay was performed in 384-well white plates (784075, Greiner, Monroe, NC) at 50  $\mu M$  CDP-star substrate in assay buffer containing 100 mM DEA–HCl (pH 9.8), 1 mM  $MgCl_2$ , and 20  $\mu M$   $ZnCl_2$ . The luminescence signal was measured after a 30-min incubation at room temperature on an EnVision plate reader (PerkinElmer, Waltham, MA). Analogous luminescent assays were optimized and used for PLAP, IAP and GCAP with the CDP-star concentration adjusted to their respective  $K_m$  values, 85  $\mu M$ , 177  $\mu M$  and 10  $\mu M$ , respectively.

### 4.3. Synthesis of compounds **1**, **3**–**20**

In a typical procedure, 2-chloro-acetophenone (0.27 mmol) in dioxane (1 mL) was added to the desired substituted amine or thiol (0.27 mmol). The solution was stirred overnight in a sealed vial at room temperature. The reaction was monitored by TLC (dichloro-

methane/methanol, 90:10, v/v) and the reaction product that precipitated from the solution was filtered, washed with a minimum amount of dioxane and then dried under high vacuum to afford the product as a solid in 30–50% yield.

Compound **1**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.55 (dd,  $J = 8.3$  and  $2.3$  Hz, 1H), 7.47 (d,  $J = 2.3$  Hz, 1H), 7.12 (s, 1H), 6.84 (d,  $J = 8.3$  Hz, 1H), 4.77 (s, 2H), 2.38 (s, 6H); LC/MS-1:  $t_R = 1.41$  (100%). MS:  $m/z$  290.9  $[M+H]^+$ , expected 291  $[M+H]^+$ .

Compound **3**:  $^1H$  NMR (DMSO)  $\delta$  7.95 (d,  $J = 3$  Hz, 2H), 7.10 (s, 1H), 6.85 (d,  $J = 3$  Hz, 2H), 4.57 (s, 2H), 2.32 (s, 6H); LC/MS-1:  $t_R = 1.83$  (100%). MS:  $m/z$  275.4  $[M+H]^+$ , expected 275.4  $[M+H]^+$ .

Compound **4**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.57 (d,  $J = 7.8$  Hz, 1H), 7.44 (s, 1H), 7.39 (d,  $J = 7.8$  Hz, 1H), 7.34 (br s, 1H), 7.10 (dd,  $J = 6.9$  and  $0.9$  Hz, 1H), 4.95 (s, 2H), 2.52 (s, 6H); LC/MS-1:  $t_R = 2.55$  (100%). MS:  $m/z$  274.9  $[M+H]^+$ , expected 275  $[M+H]^+$ .

Compound **5**:  $^1H$  NMR ( $CDCl_3$ )  $\delta$  7.72 (dd,  $J = 8.1$  and  $1.8$  Hz, 1H), 7.58 (d,  $J = 1.8$  Hz, 1H), 6.96 (d,  $J = 8.1$  Hz, 1H), 6.68 (s, 1H), 4.59 (s, 2H), 3.94 (s, 3H), 2.34 (s, 6H); LC/MS-1:  $t_R = 1.57$  (100%). MS:  $m/z$  304.9  $[M+H]^+$ , expected 305  $[M+H]^+$ .

Compound **6**:  $^1H$  NMR (DMSO)  $\delta$  7.77 (dd,  $J = 9.0$  and  $2.0$  Hz, 1H), 7.51 (d,  $J = 2.0$  Hz, 1H), 6.95 (d,  $J = 9.0$  Hz, 1H), 6.07 (s, 1H), 4.61 (s, 2H), 2.30 (s, 6H); LC/MS-1:  $t_R = 3.72$  (100%). MS:  $m/z$  303.4  $[M+H]^+$ , expected 303.5  $[M+H]^+$ .

Compound **7**:  $^1H$  NMR ( $CDCl_3$ )  $\delta$  8.05 (d,  $J = 3$  Hz, 2H), 6.95 (d,  $J = 3$  Hz, 2H), 6.71 (s, 1H), 4.57 (s, 2H), 3.87 (s, 3H), 2.32 (s, 6H); LC/MS-1:  $t_R = 3.45$  (100%). MS:  $m/z$  288.9  $[M+H]^+$ , expected 289  $[M+H]^+$ .

Compound **8**: LC/MS-1:  $t_R = 0.36$  (100%). MS:  $m/z$  219.3  $[M+H]^+$ , expected 219  $[M+H]^+$ .

Compound **9**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  8.31 (s, 2H), 7.45–7.39 (m, 2H), 6.83 (d,  $J = 9$  Hz, 1H), 4.80 (s, 2H). LC/MS-2:  $t_R = 0.65$  (100%). MS:  $m/z$  220.1  $[M+H]^+$ , expected 220  $[M+H]^+$ .

Compound **10**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.48 (dd,  $J = 8.1$  and  $1.8$  Hz, 1H), 7.42 (d,  $J = 2.1$  Hz, 1H), 7.40 (s, 1H), 7.32 (s, 1H), 6.86 (d,  $J = 8.1$  Hz, 1H), 5.56 (s, 2H), 2.10 (s, 3H);  $^{13}C$  NMR (DMSO)  $\delta$  189.6, 151.4, 145.6, 138.9, 132.7, 126.0, 121.8, 116.5, 115.2, 114.7, 58.9, 8.4; LC/MS-1:  $t_R = 2.45$  (100%). MS:  $m/z$  234.5  $[M+H]^+$ , expected 234  $[M+H]^+$ .

Compound **11**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.46–7.41 (m, 2H), 7.29–7.22 (m, 2H), 7.11–7.06 (m, 2H), 6.83 (d,  $J = 9$  Hz, 1H). MS (ESI<sup>+</sup>)  $m/z$  284.2  $(M+H)^+$ .

Compound **12**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.55–7.40 (m, 2H), 7.14 (s, 1H), 7.09 (dd,  $J = 15$  and  $1.5$  Hz, 1H), 6.83 (d,  $J = 9$  Hz, 1H), 4.80 (s, 2H), 2.34 (s, 3H); LC/MS-1:  $t_R = 0.55$  (100%). MS:  $m/z$  233.5  $[M+H]^+$ , expected 233  $[M+H]^+$ .

Compound **13**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.45–7.38 (m, 2H), 7.00 (s, 2H), 6.83 (d,  $J = 9$  Hz, 1H), 4.80 (s, 2H), 2.76 (q,  $J = 7.8$  Hz, 2H), 1.29 (t,  $J = 7.8$  Hz, 3H);  $^{13}C$  NMR (DMSO)  $\delta$  190.5, 151.8, 148.6, 145.4, 125.9, 122.8, 121.8, 119.1, 115.3, 114.8, 66.3, 19.4, 11.8; LC/MS-2:  $t_R = 0.74$  (100%). MS:  $m/z$  247.1  $[M+H]^+$ , expected 247  $[M+H]^+$ .

Compound **14**:  $^1H$  NMR ( $CD_3OD$ )  $\delta$  7.84 (d,  $J = 7.2$  Hz, 2H), 7.46–7.34 (m, 5H), 7.14 (s, 2H), 6.83 (d,  $J = 9$  Hz, 1H), 4.78 (s, 2H); LC/MS-1:  $t_R = 1.71$  (100%). MS:  $m/z$  295.4  $[M+H]^+$ , expected 295.5  $[M+H]^+$ .

Compound **15**:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.58–7.37 (m, 3H), 7.09 (br s, 1H), 6.84 (d,  $J$  = 9 Hz, 1H), 4.80 (s, 2H), 2.32 (s, 3H); MS:  $m/z$  233.3  $[\text{M}+\text{H}]^+$ , expected 233.5  $[\text{M}+\text{H}]^+$ .

Compound **16**:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.62 (s, 1H), 7.48–7.39 (m, 2H), 7.11 (s, 1H), 6.83 (d,  $J$  = 9 Hz, 1H), 4.80 (s, 2H); LC/MS-1:  $t_R$  = 1.53 (100%). MS:  $m/z$  299.6  $[\text{M}+\text{H}]^+$ , expected 299.5  $[\text{M}+\text{H}]^+$ .

Compound **17**:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.67–7.25 (m, 6H), 6.98–6.90 (m, 1H), 6.93 (d,  $J$  = 9 Hz, 1H), 6.83 (d,  $J$  = 9 Hz, 1H), 4.79 (s, 2H); MS:  $m/z$  269.3  $[\text{M}+\text{H}]^+$ , expected 269  $[\text{M}+\text{H}]^+$ .

Compound **18**:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  8.20 (s, 1H), 7.45–7.36 (m, 4H), 6.83 (d,  $J$  = 9 Hz, 1H), 4.77 (s, 2H), 2.35 (s, 6H); MS:  $m/z$  297.3  $[\text{M}+\text{H}]^+$ , expected 297.5  $[\text{M}+\text{H}]^+$ .

Compound **19**:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.45–7.40 (m, 2H), 6.93 (s, 1H), 6.83 (d,  $J$  = 9 Hz, 1H), 4.79 (s, 2H), 2.32 (s, 3H); LC/MS-1:  $t_R$  = 1.48 (100%). MS:  $m/z$  313.0  $[\text{M}+\text{H}]^+$ , expected 313  $[\text{M}+\text{H}]^+$ .

Compound **20**:  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.90–7.39 (m, 5H), 6.98–6.90 (m, 1H), 6.93 (d,  $J$  = 6 Hz, 1H), 6.83 (d,  $J$  = 9 Hz, 1H), 4.79 (s, 2H), 2.68 (s, 3H);  $^{13}\text{C}$  NMR ( $\text{DMSO}$ )  $\delta$  189.6, 152.7, 151.4, 151.2, 145.4, 135.4, 126.1, 122.9, 122.3, 121.9, 117.3, 115.2, 113.9, 110.2, 66.3, 13.5; MS:  $m/z$  283.3  $[\text{M}+\text{H}]^+$ , expected 283  $[\text{M}+\text{H}]^+$ .

Compound **22**: 50 mg (0.36 mmol) of 3,4-dihydroxybenzaldehyde and 44 mg (0.36 mmol) of 2-amino-4,6-dimethylpyridine was stirred in 1 mL DCE in the presence of 153 mg (0.72 mmol) of sodium triacetoxymethylborohydride and 1 drop of acetic acid at rt overnight. The reaction was quenched with 1 mL water. The product was extracted with ethyl acetate and purified by PTLC (ethyl acetate/methanol, 90:10, v/v) to give 25 mg of the product (28% yield).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  7.29 (s, 1H), 6.88 (d,  $J$  = 9 Hz, 1H), 6.80–6.62 (m, 2H), 4.41 (s, 2H), 2.25 (s, 3H), 1.96 (s, 3H); LC/MS-1:  $t_R$  = 2.33 (85.13%). MS:  $m/z$  246.4  $[\text{M}+\text{H}]^+$ , expected 246.5  $[\text{M}+\text{H}]^+$ .

Compound **25**: 50 mg (0.23 mmol) of 3,4-dihydroxybenzylamine **23** was dissolved in 1 mL THF/1 mL DMF. 100 mg (0.27 mmol) of HBTU, 128  $\mu\text{L}$  (0.69 mmol) of DIEA and 32 mg (0.23 mmol) of 1,5-dimethyl-3-furoic acid were added and the mixture was stirred at rt overnight. The crude material was purified by PTLC (DCM/MeOH, 90:10, v/v) to give a yellow solid (60 mg, 98% yield).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  6.88 (s, 1H), 6.76 (s, 1H), 6.69 (d,  $J$  = 7.8 Hz, 1H), 6.63 (d,  $J$  = 7.8 Hz, 1H), 6.27 (s, 1H), 4.35 (s, 2H), 2.25 (s, 3H), 1.96 (s, 3H); LC/MS-1:  $t_R$  = 2.33 (100%). MS:  $m/z$  262.5  $[\text{M}+\text{H}]^+$ , expected 262.5  $[\text{M}+\text{H}]^+$ .

Compound **26**: 44 mg (0.23 mmol) of dopamine-HCl **24** was dissolved in 1 mL THF/1 mL DMF. 100 mg (0.27 mmol) of HBTU, 128  $\mu\text{L}$  (0.69 mmol) of DIEA and 32 mg (0.23 mmol) of 4,5-dimethyl-2-furoic acid were added and the mixture was stirred at rt overnight. The crude material was purified by PTLC (DCM/MeOH, 90:10, v/v) to give an off-white solid (30 mg, 47% yield).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  6.68 (d,  $J$  = 8 Hz, 1H), 6.66 (s, 1H), 6.54 (d,  $J$  = 9.4 Hz, 1H), 6.18 (s, 1H), 3.39–3.49 (m, 2H), 2.69 (t,  $J$  = 8 Hz, 2H), 2.44 (s, 3H), 2.21 (s, 3H); LC/MS-1:  $t_R$  = 2.23 (100%). MS:  $m/z$  275.9  $[\text{M}+\text{H}]^+$ , expected 276  $[\text{M}+\text{H}]^+$ .

Compounds **27–30** and **32–33** were purchased from ChemBridge and **31** from ChemDiv.

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