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Design, Synthesis and Evaluation of Benzoisothiazolones as Selective Inhibitors of PHOSPHO1

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Abstract

We report the discovery and characterization of a series of benzisothiazolone inhibitors of PHOSPHO1, a newly identified soluble phosphatase implicated in skeletal mineralization and soft tissue ossification abnormalities. High-throughput screening (HTS) of a small molecule library led to the identification of benzoisothiazolones as potent and selective inhibitors of PHOSPHO1. Critical structural requirements for activity were determined, and the compounds were subsequently derivatized and measured for in vitro activity and ADME parameters including metabolic stability and permeability. On the basis of its overall profile the benzoisothiazolone analogue 2q was selected as MLPCN probe ML086

Keywords

Phosphatase; Vascular calcification; Probe compound; ML086; PHOSPHO1

PHOSPHO1 is a recently identified orphan phosphatase that belongs to the family of halo-acid dehalogenases. It is a soluble phosphatase with specificity for phosphoethanolamine (P-Etn) and phosphocholine (P-Cho) present in matrix vesicles (MVs).[1] PHOSPHO1 is responsible for increasing the local concentration of inorganic phosphate (Pi) inside MVs to change the phosphate:pyrophosphate (Pi/PPi) ratio to favor precipitation of hydroxyapatite (HA) seed crystals.[2] Aberrations of the Pi/PPi ratio have been associated with numerous

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pathologies. Low extracellular PPi (ePPi) production has been identified as a cause in the development of severe medial vascular calcification (MVC) known as generalized arterial calcification of infancy (GACI; OMIM # 208000)[3] as well as ossification of the posterior longitudinal ligaments of the spine (OPLL; OMIM # 602475) and osteoarthritis (OA).[4] In addition, low ePPi transport manifests as ankylosing vertebral hyperostosis (DISH; OMIM # 106400), chondrocalcinosis (OMIM # 605145) and ankylosing spondylitis (AS; OMIM # 106300).[5] On the other hand, accumulation of ePPi results in rickets or osteomalacia, known as hypophosphatasia (HPP; OMIM # 171760).[6] Because of the accumulation of ePPi, HPP patients may also display chondrocalcinosis or calcium pyrophosphate dihydrate deposition (CPPD) disease (OMIM # 118600). However, many of these conditions have been linked to deficiencies in other transporters and phosphatases, most notably tissue-nonspecific alkaline phosphatase (TNAP) that is also found in the same biological compartment.[7] There is therefore a significant need to identify small molecule compounds that can probe the function of these enzymes and provide a starting point for the development of therapeutic agents.

We previously reported the synthesis and optimization of selective small molecule inhibitors of TNAP that have been employed to investigate the role of TNAP in vascular calcification.[8] We also disclosed the synthesis and characterization of a series of compounds that inhibit phosphomannose isomerase (PMI), an enzyme implicated in therapeutically important protein glycosylation processes.[8a, 9] These small molecule probes, discovered using high-throughput screening (HTS) and chemical optimization through the Molecular Libraries Probe Production Centers Network (MLPCN; http://mli.nih.gov/mli/mlpcn/), have found utility for investigating the role of intracellular and extracellular phosphatases. The structure of the PMI inhibitor probe ML089 (**1**) is shown in Figure 1. We hypothesized that, in a similar manner, it would be possible to discover small molecule inhibitors of PHOSPHO1 that would help to delineate the role of this enzyme in skeletal mineralization and soft tissue ossification abnormalities at a fundamental level. Furthermore, selective PHOSPHO1 inhibitors would be useful tools to elucidate the mechanism of action for the aforementioned diseases. With this in mind a high-throughput screening (HTS) campaign was performed through the MLPCN.

The screen employed a colorimetric assay based on the ability of PHOSPHO1 to liberate phosphate from P-Etn and its reaction with the Biomol Green reagent (Biomol International, Plymouth Meeting, PA, USA). The construct was designed to express PHOSPHO1 protein fused to a V5 epitope and 6 His-tag at the C-terminus. A diverse library of 288,481 compounds from the MLSMR collection was tested at a single concentration of 13.3 μM (PubChem AID 1565). This provided 3,164 compounds that showed greater than 60% activity in the single point assay, a hit rate of 1.1%. Hit confirmation was performed using the colorimetric assay to verify inhibitory activity against PHOSPHO1 in dose-response mode performed in duplicate using a 10-point 2-fold serial dilution of the hit compounds in DMSO. Inhibitors that were active in dose-response mode against PHOSPHO1 and soluble in the concentration range relevant to their potency were classified as confirmed hits. This led to the identification of several sub-micromolar inhibitors of PHOSPHO1 (see PubChem link to AID 1565 and 1666 for details).

It was noted that some of the confirmed hits fell into the benzoisothiazolone class of small molecules, such as **1,** that were previously identified as PMI inhibitors. For example, the unsubstituted benzoisothiazolone **2a** (Table 1) inhibited PHOSPHO1 with an IC_{50} value of 0.94μM while also inhibiting PMI (IC_{50} = 6.4 μM). We hypothesized that within this series it might be possible to optimize the potency of compounds against PHOSPHO1 while reducing or eliminating activity at PMI and PMM2. The benzothiazolone series was therefore prioritized for chemical optimization using both analogue by catalogue (ABC) and synthetic chemistry.

Analogues of the benzoisothiazolone hits were synthesized by a method originally reported by Correa *et al.*[10] that utilized a key cyclization step using phenyliodine bis(trifluoroacetate) (PIFA) to generate a N-acylnitrenium ion followed by intramolecular trapping by sulfur (Scheme 1a). The synthesis of the analogues was carried out in a semi-convergent manner. The required aniline precursors were either purchased or, in the case of the amides, prepared via EDCI mediated coupling of amines with the appropriate benzoic acid derivatives (Scheme 1b).[11] This product was then coupled with methyl 2-mercaptobenzoate and subjected to the cyclization conditions (Scheme 1a). This synthetic methodology allowed for preparation of the benzoisothiazolone derivatives shown in Tables 1 and 2.[12]

The potency and selectivity of the benzoisothiazolone analogues were assessed by *in vitro* enzymatic assays using purified human PHOSPHO1, PMI, or PMM2[13] to establish a preliminary SAR. As noted previously, the hit compound **2a** inhibited PHOSPHO1 with good potency but also significantly inhibits PMI (Table 1). Fluoro substitution (R^1 = F) of the benzoisothiazolone moiety, as in **2b,** provides a marginal improvement in potency at PHOSPHO1 but also increased potency at PMI (IC_{50} = 1.3 μM). Monomethyl substitution of the phenyl ring, as in **2c** and **2d**, lessened potency at PHOSPHO1 while retaining the unwanted activity at PMI. Substitution at the 4-position of the phenyl ring with methoxy (**2e**), fluoro (**2f**, **2g**), or NMe_2 (**2h**) effectively worsened potency against PHOSPHO1 while in general increasing potency at PMI. Substitution at the 3-position with chloro, as in **2i**, provided a compound with similar potency at both PHOSPHO1 and PMI. Interestingly, none of the initial set of compounds **2a**-**2i** had significant activity at PMM2. The 2,5- or 2,3-dimethyl substitution patterns (**2j** and **2l**, respectively) gave compounds that were similarly potent at both PHOSPHO1 and PMI. Introduction of R^1= fluoro, as in **2k**, unfortunately enhanced potency at both PMI and PMM2. In contrast to **2i**, the 3-chloro-4-fluorophenyl derivative **2m** was potent at PHOSPHO1 but inactive at both PMI and PMM2. Carboxylic acid substitution at the 3-position (**2n**) provided a potent PHOSPHO1 inhibitor with micromolar activity at PMI and no activity at PMM2. The methyl ester derivative **2o** exhibited sub-micromolar potency at PHOSPHO1 but also micromolar activity at both PMI and PMM2, whereas the ethyl ester derivative **2p** was essentially devoid of activity at PMI and PMM2. The breakthrough came, however, with the dimethyl amide derivative **2q**, exhibiting an IC_{50} value of 140 nM at PHOSPHO1 and no activity at PMI or PMM2. Interestingly, the corresponding benzylamide derivative **2r**, while potent at PHOSPHO1 also showed activity at PMI and PMM2.

Based on the promising data for the first set of compounds, and in particular **2q**, we next tested a series of analogues containing a sulfonamide moiety at the 3-position of the phenyl ring. The results of these efforts are shown in Table 2. Several analogues in this series exhibited good potency as PHOSPHO1 inhibitors, with the dimethyl (**2s**) and diethyl (**2t**) analogues being especially potent. Interestingly, while compound **2s** was active at PMI and PMM2 at micromolar levels, the diethyl sulfonamide **2t** was devoid of activity at these phosphatases. The anthranilic acid sulfonamide **2u** exhibited submicromolar potency at both PHOSPHO1 and PMI. Sulfonamide derivatives **2v-2y** were less potent at PHOSPHO1 and all had some level of activity at PMI and PMM2.

Select PHOSPHO1 inhibitors (**2n, 2o, 2q, 2s**) were comprehensively profiled in *in vitro* absorption, distribution, metabolism and excretion (ADME) assays (Table 3).[14] The data in Table 3 provide insight into the drug-likeness and potential for systemic activity of compounds, thus enabling advanced testing and future target validation efforts. The selected compounds were shown to have properties indicative of the potential for oral availability including acceptable metabolic stability, good permeability across artificial lipid membranes, and good solubility. No significant cell toxicity could be detected for any of the analogues. With these data indicative of drug-like behavior, good potency and selectivity, this series may be suitable for *in vivo* proof-of-concept studies. Of note is the esterification of the carboxylic group on **2o**, which essentially forms a pro-drug prone to facile metabolic cleavage and subsequent formation of **2n**. Since **2o** exhibits improved permeability parameters compared with **2n**, the data suggest that the development of a series of pro-drug analogues may be a viable approach to develop compounds with *in vivo* activity.

Compound **2q** was additionally tested for selectivity against the enzymes TNAP and ectonucleotide pyrophosphatase/phosphodiesterase-1 (NPP-1) (Table 4). Gratifyingly, **2q** was found to be devoid of any significant inhibitory activity against these counter targets. With respect to testing in other screens through the MLPCN program, **2q** (CID16749996) was found to have weak activity in a screen for inhibitors of the mevalonate pathway in streptococcus pneumonia (AID1028). As shown in in Table 4, **2q** was at least 170-fold selective for PHOSPHO1 versus counter targets.

In conclusion, we have identified and developed a series of PHOSPHO1 inhibitors with sub-micromolar potencies and promising drug-like properties. Medicinal chemistry efforts were applied to the optimization of a benzoisothialozone core scaffold initially identified through HTS. A carefully guided SAR study yielded a series of highly active inhibitors with proven ability to inhibit PHOSPHO1 in *ex vivo* models.[15] On the basis of its overall profile compound **2q** was selected as MLPCN probe ML086. This work provides an example of a successful strategy using medicinal chemistry to develop a useful biological probe. Furthermore, the drug-like properties of the resulting compounds provide an opportunity to lay the foundation for the development of therapeutic agents suitable for the treatment of diseases caused by MVC.

Acknowledgments

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12. General synthesis of PHOSPHO1 inhibitors: To a stirred solution of benzoic acid derivative 5 (300mg, 2.18 mmol) in CH_2Cl_2 was added an amine (266 mg, 3.27 mmol) followed by the coupling agents; HOBT (442 mg, 3.27 mmol) and EDCI (630 mg, 3.27 mmol) and then Hunig's base (1.14 mL, 6.54 mmol). The reaction was stirred at room temperature over night, then quenched with saturated $NaHCO_3$ solution and CH_2Cl_2, then dried over Na_2SO_4. The solvents were removed by rotary evaporation, no further purification was needed. To a stirred solution of the aniline 6 (260 mg, 1.58 mmol) in CH_2Cl_2 at 0 °C under nitrogen was added $AlMe_3$ (2 mL, 1 M in THF) dropwise and the reaction was slowly warmed to room temperature. The mixture was stirred continuously for an additional 30 min. Methyl thiosalicylate (130 µL, 0.790 mmol) was added and the reaction was heated to 60 °C and then heated under reflux overnight. The reaction was quenched with HCl (5% aq.) and CH_2Cl_2 was added (50 mL). The organic layer was separated and washed with saturated $NaHCO_3$ solution, then brine, and dried over Na_2SO_4. The solvents were removed by rotary evaporation. The products were purified by flash chromatography or reverse phase HPLC and lyophilized to provide thiols 4 which were determined to be > 95% pure by HPLC-UV, HPLC-MS, and ^{1}H NMR. A solution of PIFA in CH_2Cl_2 was added to 0 °C solution of the benzamide (250 mg, 0.833 mmol) and TFA (0.185 mL, 16 M) in CH_2Cl_2. The reaction mixture was stirred at room temperature overnight. The solvents were removed in vacuo and the product isolated by flash chromatography or reverse phase HPLC and lyophilized to provide the final compounds (2) which were determined to be > 95% pure by HPLC-UV, HPLC-MS, and ^{1}H NMR.

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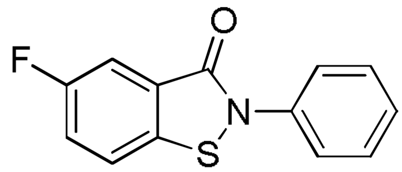
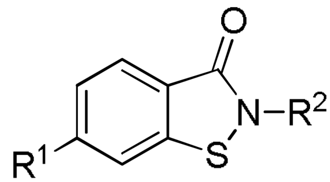


1 ML089



2 R^1 = H, F
R^2 = aryl

Figure 1.
Phosphomannose isomerase (PMI) probe ML089 (**1**) and the benzothiazolone scaffold of the PHOSPHO1 screening hits.

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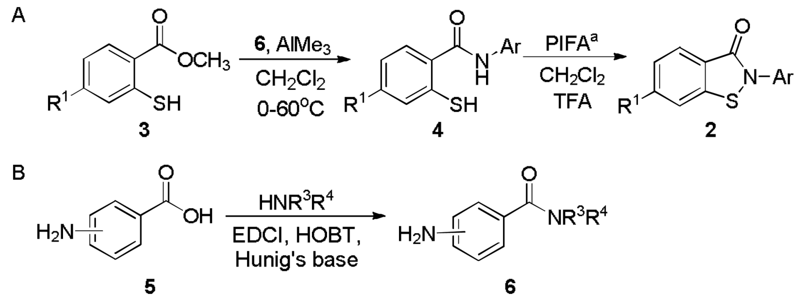
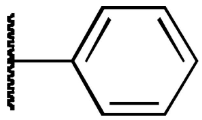
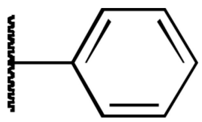
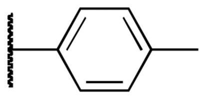
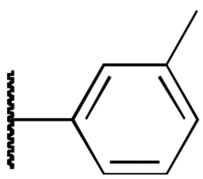
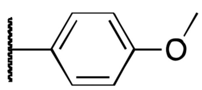
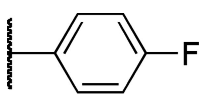
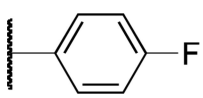
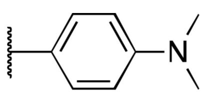
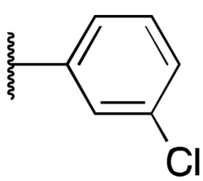


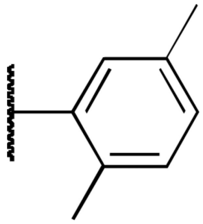
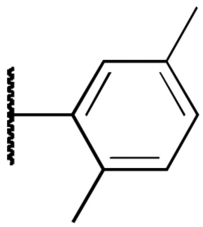
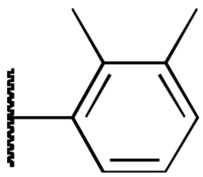
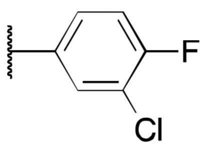
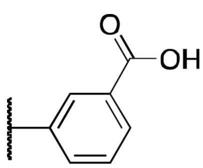
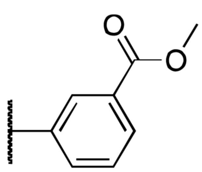
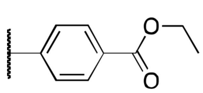
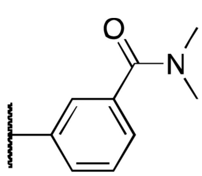
Scheme 1.

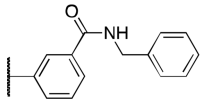
General synthetic sequences for the synthesis of small molecule PHOSPHO1 inhibitors. [a]PIFA phenyliodine bis(trifluoroacetate).

Table 1

Summary of first round of SAR for compounds synthesized and tested for PHOSPHO1 inhibition as well as selectivity against related phosphatases.

Cmpd.	Ar	R^1	PHOSPHO1[a]	PMI[b] IC50 [μM]	PMM2[b]
2a		H	0.94	6.4	> 20
2b		F	0.79	1.3	> 50
2c		H	1.3	3.9	> 30
2d		H	2.7	6.0	> 20
2e		F	6.7	1.0	> 10
2f		H	4.9	3.4	> 20
2g		F	> 10	3.6	> 50
2h		H	11	8.4	> 30
2i		H	5.2	4.8	> 30

Cmpd.	Ar	R[1]	PHOSPHO1[a]	PMI[b] IC50 [μM]	PMM2[b]
2j		H	4.0	6.6	> 20
2k		F	3.3	1.1	7.3
21		F	4.7	9.9	> 20
2m		H	1.8	> 50	> 100
2n		H	1.1	3.3	52
2o		H	0.82	4.9	12
2p		H	1.2	23	> 50
2q		H	0.14	> 50	> 50

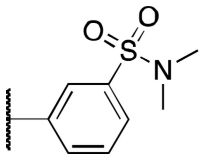
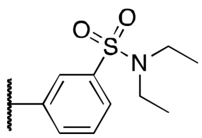
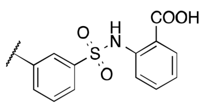
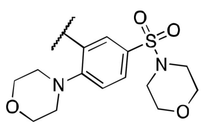
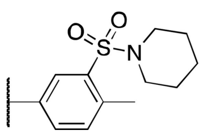
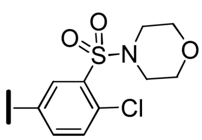
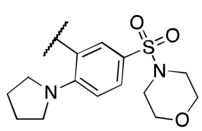
Cmpd.	Ar	R^1	PHOSPHO1[a]	PMI[b] IC50 [μM]	PMM2[b]
2r		H	2.3	2.3	18

[a]See http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1666 for details of assay protocol.

[b]See Dahl *et al.*, 2011[8a] for assay protocol.

Table 2

Data for analogues with a sulfonamide substituent at the 3-position of the phenyl ring.

Cmpd.	Ar	PHOSPHO1[a]	PMI[b] IC_{50} [μM]	PMM2[b]
2s		0.50	2.8	13
2t		0.56	> 50	> 100
2u		0.81	0.71	5.2
2v		1.2	2.6	9.9
2w		1.8	3.1	11
2x		1.1	8.6	63
2y		7.5	7.5	30

[a]See http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1666 for details of assay protocol.

[b]See Dahl *et al.*, 2011[8a] for assay protocol.

Table 3

Characterization of a selection of synthesized analogues for drug-like properties in a range of *in vitro* ADME assays. For details of assay protocols see Khan *et al., 2011* [14]

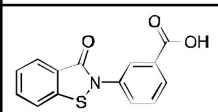
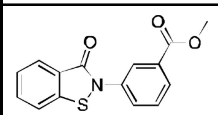
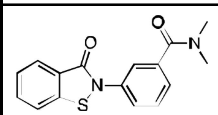
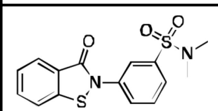
Cmpd.	Structure	Aqueous Solubility in pION's buffer [µg/ml]			PAMPA Permeability [$\times10^{-6}$ cm/s] Acceptor pH 7.4			Plasma Protein Binding [% Bound]		Plasma Stability [% Remaining at 3hrs] Plasma: 1× PBS, pH 7.4, 1:1 1×PBS, pH 7.4		Hepatic Microsome Stability [% Remaining at 1hr] (NADPH minus)		Toxicity towards Fa2N-4 Immortalized Human Hepatocytes LC_{50} [µM]
		PH 5.0	PH 6.2	PH 7.4	PH 5.0	PH 6.2	PH 7.4	Human 1 µM / 10 µM	Mouse 1 µM / 10 µM	Human	Mouse	Human	Mouse	
2n		> 27	> 27	> 27	157	18	<4.4	n.d. / 59.4	18.8 /30.1	50.2 55.5	100 71.2	100	100	> 50
2o		12.6	18.9	20.8	1294	1231	1300	41.2 / 57.3	48.0 / 37.6	15.4 49.3	59.6 55.2	72.4 31.5	45.9 44.6	> 50
2q		> 30	> 30	> 30	94	92	97	41.3 /44.2	48.0 / 46.7	18.9 60.0	76.2 56.9	45.7	56.6	> 50
2s		23.4	24.6	23.8	781	817	777	n.d. / 56.5	35.6 / 34.1	11.4 32.3	65.3 40.0	72.4	100	> 50

Table 4

Activity of 2q (MLPCN Probe ML-086) on the target (PHOSPHO1) compared with counter-target enzymes and a pathway screen.

	PHOSPHO1	TNAP	NPP-1	PMI	PMM2	AID1028[a]
IC_{50} [μM]	0.14	> 100	> 30	62	76	24
Selectivity	-	> 719	> 215	442	549	176

[a] http://pubchem.ncbi.nlmnih.gov/assay/assay.cgi?aid=1028