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Structure-Based Optimization of Benzoic Acids as Inhibitors of Protein Tyrosine Phosphatase 1B and Low Molecular Weight Protein Tyrosine Phosphatase

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Protein tyrosine phosphorylation plays a key role in the regulation of signal transduction, which underlies many fundamental cellular functions. The cellular level of protein tyrosine phosphorylation is the result of the concerted activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTPs comprise a large family of more than 100 enzymes, which can function both as positive and negative modulators in specific signaling pathways, thus playing complex and crucial physiological roles in most mammalian cells.[1]

As observed with PTKs, the defective or inappropriate functioning of PTPs has been recognized as being linked to the development of many human pathologies, including diabetes, obesity, and cancer. Thus, PTPs have become the focus of increasing interest as novel molecular targets in drug design.[2]

PTP1B is an intracellular PTP which acts as a major negative regulator of both insulin and leptin signaling pathways.[3–5] Low molecular weight PTP (LMW-PTP) also acts as a negative regulator of insulin mediated mitotic and metabolic signaling by producing effects which are distinct and independent of those of PTP1B.[6–8] There is compelling evidence that PTP1B aberrant expression or dysfunction is linked to the development of insulin resistance, which is the biochemical defect underlying related, even co-morbid, metabolic pathologies, such as type 2 diabetes (DM2), obesity, and metabolic syndrome, and currently available therapeutic treatments are not adequate to fight insulin resistance.[9,10] PTP1B knockout mice exhibit increased insulin and leptin sensitivity with resistance to diet-induced obesity and are otherwise normal.[4,11] It has also been demonstrated that knocking down PTP1B or LMW-PTP by means of antisense oligonucleotides increases insulin sensitivity in animal models and humans.[8,12] Thus, PTP1B has emerged as a tried and tested molecular target for the development of novel insulin-sensitizer agents addressing both DM2 and obesity.[13–16] Inhibitors of both PTP1B and LMW-PTP could also show promise by counteracting insulin resistance through distinct signaling pathways.

PTP1B and LMW-PTP have also been implicated in oncogenesis and are recognized as positive factors in the progression of some types of human cancer. Therefore, they can also be attractive targets for the development of novel antitumor drugs.[17–20]

The identification of selective, safe, and orally available small-molecule PTP inhibitors has proven challenging. This is due to the highly conserved and charged nature of the PTP active site that can affect both selectivity and bioavailability. However, the presence of unique features in the loops bordering the catalytic site of each PTP can be used for the structure-based design of selective inhibitors. In PTP1B, the catalytic domain is completed by two loops which converge around it; the flexible WPD loop (residues 179–189) and the YRD loop (residues 46–48). These are both implicated in substrate and inhibitor affinity and specificity.[1e,13–15,21] A secondary, noncatalytic, arylphosphate binding site located close to the PTP1B active site has been identified and this is lined by Tyr20, Arg24, Ala27, Phe52, Arg254, Met258, and Gly259. It is not present in all PTPs, thus providing a structural basis for the targeted design of selective bidentate inhibitors that can simultaneously occupy both the active site and the nearby noncatalytic site.[1e,13,15,21,22] Human LMW-PTP possesses a catalytic phosphate binding site that can be structurally superimposed on that of PTP1B and it is expressed in two isoforms (IF1 and IF2) which differ only in the 40–73 sequence. This "variable loop" surrounds the catalytic site and appears to be important for the specific binding to the two isoenzymes.[1e,23,24]

Recently, we reported a series of 4-[(5-arylidene-2,4-dioxothiazolidin-3-yl)methyl]benzoic acids **1** designed as small-molecule nonphosphorous PTP1B and LMW-PTP inhibitors in which the *p*-methylbenzoic acid residue attached to N3 can act as a phosphotyrosine (pTyr) mimic by replicating the interactions of pTyr with the catalytic site of the enzyme.[25] In fact, inserting monoanionic pTyr mimics into optimal templates is considered to be a valid method to obtain inhibitors with low polarity.[2c,14,26] Compounds **1** were found to be effective PTP inhibitors with an appreciable selectivity toward human PTP1B and the IF1 isoform of human LMW-PTP. A 5-arylidene moiety containing two aromatic rings promoted their inhibitory activity,

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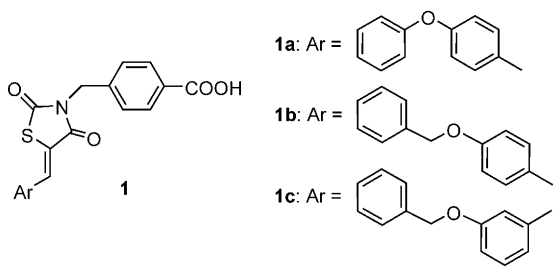


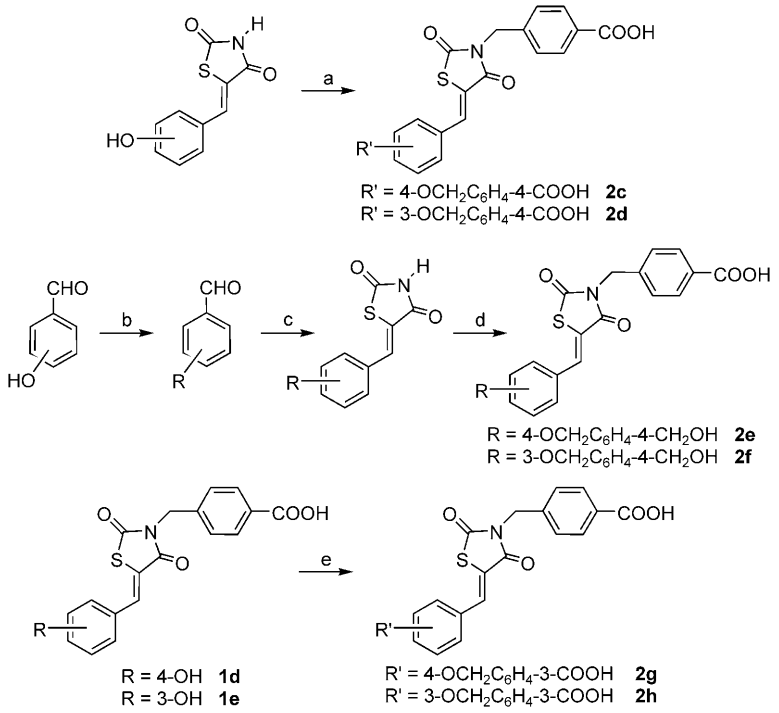
particularly toward PTP1B and IF1.[25] Replacing the carbonyl group in position 2 of the thiazolidinedione scaffold with a phenylimino moiety provided an appreciable gain in potency in some cases.[27]

Compounds **1a** and **1b** were found to be potent inhibitors of both human PTP1B and IF1 and, to the best of our knowledge, **1a** is the most effective in vitro inhibitor of human LMW-PTP (IC_{50} = 0.9 μM toward IF1) reported to date.[25] Moreover, in agreement with our in vitro inhibition data, molecular docking experiments into the PTP1B active site indicated that **1a** and **1b** can act as dual site PTP1B inhibitors as their 5-arylidene moieties fit the noncatalytic arylphosphate binding site very well.[25]

In pursuing our research, we designed structural modifications of the 5-arylidene moiety aimed to enhance both the potency and selectivity of these PTP1B/LMW-PTP inhibitors. We decided to maintain the benzyloxybenzylidene moiety, which proved to be related to high inhibition levels, and to insert substituents onto it to improve the interaction with amino acid residues flanking the PTP1B and LMW-PTP catalytic sites. In particular, the introduction of a second pTyr mimic group could enhance the affinity of these bidentate inhibitors for the secondary noncatalytic pocket of PTP1B.

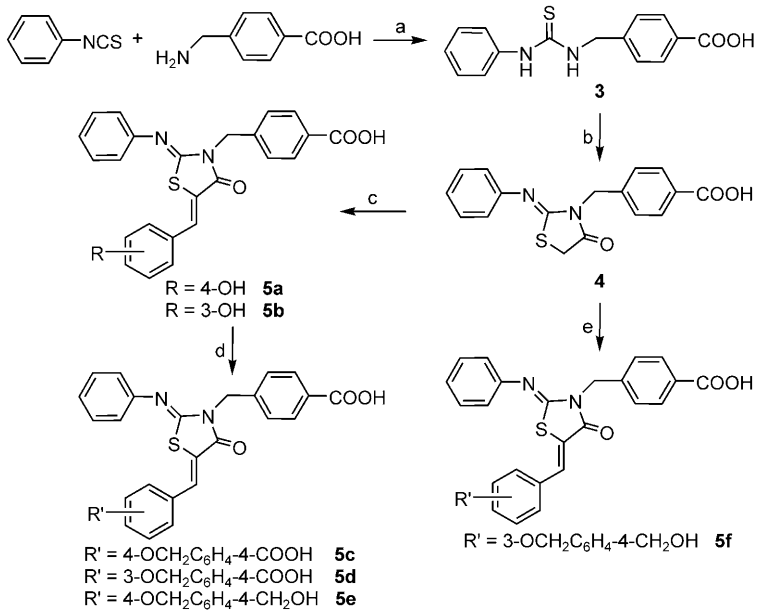
Compounds **2c–h** and **5c–f** were prepared following the synthetic routes depicted in Schemes 1 and 2. The microwave-assisted N/O-alkylation of 5-(3/4-hydroxybenzylidene)-2,4-thiazolidinediones with 4-(bromomethyl)benzoic acid led to derivatives **2c** and **2d**. Analogously, the O-alkylation of **1d** and **1e**[25] with 3-(bromomethyl)benzoic acid produced **2g** and **2h** (Scheme 1). The preparation of acids **2e** and **2f** started from the synthesis of the appropriate aldehydes which were condensed with commercial 2,4-thiazolidinedione, followed by N-alkylation with 4-(bromomethyl)benzoic acid (Scheme 1). The synthesis of analogues **5** was accomplished starting from the preparation of thiourea **3**, which reacted with chloroacetyl chloride to provide acid **4**. The subsequent microwave-assisted condensation of **4** with suitable aromatic aldehydes provided compounds **5a**, **5b**, and **5f**. The O-alkylation of **5a** and **5b** led to derivatives **5c–e** (Scheme 2).

Benzoic acids **2c–h** and **5c–f** were tested for their in vitro inhibitory activity against recombinant human PTP1B, IF1 and IF2 isoforms of human LMW-PTP, and the LMW-PTP from *S. cerevisiae* (Ltp1). Compounds **5c** and **5d** were also evaluated for other PTPs (YopH, LAR, PTPβ).



Scheme 1. Synthesis of 5-arylidene-2,4-thiazolidinediones **2c–h**. *Reagents and conditions:* a) 1) 4-$BrCH_2C_6H_4COOH$, DMF, NaH, MW 125 °C, 30 min, 2) HCl, 45–70%; b) 4-$ClCH_2C_6H_4CH_2OH$, DMF, NaH, MW 125 °C, 15 min, 30–47%; c) 2,4-thiazolidinedione, $C_5H_{11}N$, EtOH, Δ, 21–30%; d) 1) 4-$BrCH_2C_6H_4COOH$, K_2CO_3, acetone, Δ, 2) HCl, 52–85%; e) 1) 3-$BrCH_2C_6H_4COOH$, DMF, NaH, MW 125 °C, 30 min, 2) HCl, 30–61%.

All the new derivatives were shown to be potent PTP1B inhibitors with low-micromolar or sub-micromolar IC_{50} values (Table 1). The introduction of a carboxy group in the *para* position of the benzyloxy moiety in **2c**, **2d** and **5c**, **5d** provided a significant gain in potency with respect to the parent compounds (**1b**, **1c** and **6b**, **6c**), which was particularly marked for **2c** (sevenfold) and **5d** (17-fold). The displacement of the carboxy group of compounds **2c** and **2d** to the *meta* position (analogues **2g** and **2h**) produced a 7–12-fold decrease in the inhibitory effect (Table 1). Replacing the carboxylate in the *para* position of compounds **2c**, **2d** and **5c**, **5d** with a nonionizable hydroxymethyl group resulted in less active derivatives (**2e**, **2f** and **5e**, **5f**) which, however, maintained appreciable low-micromolar IC_{50} values (Table 1). Comparing the activity of 2,4-thiazolidinediones **2** with corresponding analogues **5** also highlighted that the replacement of the carbonyl group in



Scheme 2. Synthesis of 5-arylidene-2-phenylimino-4-thiazolidinones **5c–f**. *Reagents and conditions:* a) EtOH, Δ, 24 h, 100%; b) $ClCH_2COCl$, Et_3N, EtOH, Δ, 24 h, 70%; c) 3- or 4-hydroxybenzaldehyde, $C_5H_{11}N$, EtOH, MW 140 °C, 6 h, 62–67%; d) 4-$BrCH_2C_6H_4COOH$ or 4-$ClCH_2C_6H_4CH_2OH$, DMF, NaH, MW 125 °C, 30 min, 30–48%; e) 3-{[4-(hydroxymethyl)-phenyl]methoxy}benzaldehyde, $C_5H_{11}N$, EtOH, MW 140 °C, 6 h, 35%.

position 2 of the five-membered heterocyclic scaffold with a phenylimino moiety can result in different effects on PTP1B inhibitory potency. Compounds **2c** and **2f** were in fact shown to be two- to threefold more potent inhibitors than analogues **5c** and **5f**. In contrast, **5d** was threefold more effective than **2d**, whereas **2e** and **5e** displayed very similar activity levels.

The most effective inhibitors (**2c**, **2d** and **5c**, **5d**) were assayed for reversibility (Figure 1) and inhibition type (see Supporting Information). Compounds **2c** and **5d** are mixed-type inhibitors, whereas **2d** and **5c** are competitive inhibitors; however, all are reversible inhibitors.

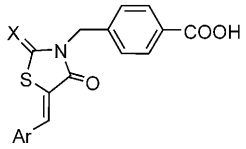
The results of the evaluation of compounds **2c–h** and **5c–f** as LMW-PTP inhibitors (Figure 2) showed that compounds **2c**, **5c**, and **5d** greatly decrease IF1 enzyme activity, but are less effective toward IF2 and Lpt1. Panels B) and C) (IF2 and Ltp1, Figure 2) refer to assays performed at inhibitor doses 20-fold higher than those used for IF1, clearly displaying the selectivity of our inhibitors toward IF1 over IF2 and Ltp1. This was particularly noticeable for compound **5d**, which proved to be 55-fold more effective against IF1 than against IF2 (Table 2). As observed for PTP1B, the shift of the carboxy group from the *para* to the *meta* position or its replacement with a hydroxymethyl group led to a generally significant decrease in IF1 inhibition levels. Another point to note is that attaching a 2-phenylimino moiety to the 4-thiazolidinone ring (**5c**, **5d**) enhanced the activity toward IF1 with respect to analogues **2c**, **2d** (Figure 2).

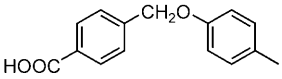
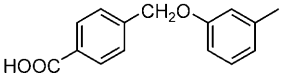
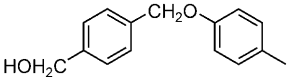
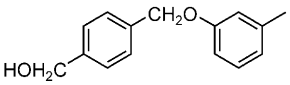
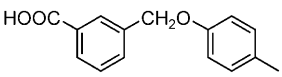
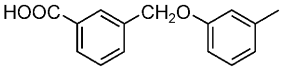
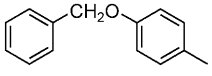
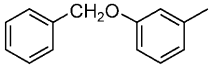
Compounds **5c** and **5d** were also tested against YopH, LAR, and PTPβ (Table 2). Both of them proved to be potent inhibitors of human PTPβ, which is emerging as a possible biological target in the design of new cardiovascular and antitumor drugs.[28] However, compounds **5c** and **5d** are less active toward LAR and YopH.

Our inhibition data suggested that, as expected, the *p*-methylbenzoic acid residue attached to the 5-phenoxymethylidene moiety of **2c**, **2d** and **5c**, **5d** could act as a pTyr mimic. It is also likely that residues of the secondary noncatalytic pocket

Table 1. In vitro inhibitory activity of compounds **2c–h** and **5c–f** against human PTP1B.

1, **2**: X = O
5, **6**: X = NPh



Ar	Compd	IC_{50} [μM][a]	Compd	IC_{50} [μM][a]
	**2c**	0.24 ± 0.07	**5c**	0.55 ± 0.01
	**2d**	0.63 ± 0.15	**5d**	0.22 ± 0.01
	**2e**	2.83 ± 0.16	**5e**	3.18 ± 0.67
	**2f**	1.34 ± 0.43	**5f**	4.26 ± 0.28
	**2g**	2.90 ± 0.10	–	–
	**2h**	4.30 ± 0.20	–	–
	**1b**[b]	1.6 ± 0.2	**6b**[c]	1.1 ± 0.1
	**1c**[b]	1.1 ± 0.1	**6c**[c]	3.8 ± 0.1

[a] IC_{50} values were determined by regression analyses and are expressed as the mean ± SE of three replicates. [b] Ref. [25]. [c] Ref. [27].

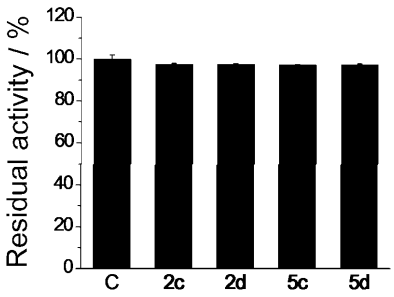


Figure 1. Inhibition reversibility assay. Aliquots of PTP1B were incubated in the presence of each compound at 50 μM for 1 h at 37 °C. The enzyme was then diluted 400-fold with assay buffer to measure the residual activity (37 °C, 40 mM *p*-nitrophenyl phosphate). Control experiments (C) were carried out by adding DMSO. All tests were performed in triplicate; data represent the mean ± SE.

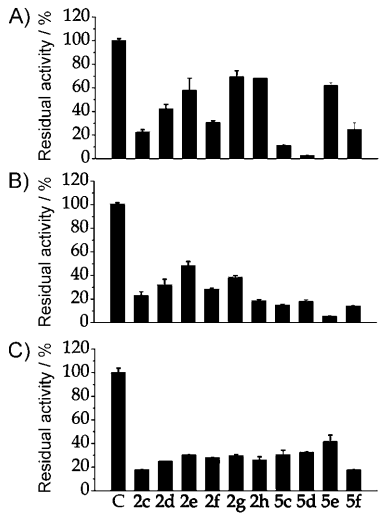


Figure 2. In vitro inhibitory activity of compounds **2c**–**h** and **5c**–**f** against LMW-PTPs. Tests were performed at pH 7.0 and 37 °C using a fixed substrate concentration, 2 mM *p*-nitrophenyl phosphate. The final concentration for each inhibitor was: A) IFI: 2.5 μM; B) IF2: 50 μM; C) Ltp1: 50 μM. Control experiments (C) were carried out using DMSO. All tests were performed in triplicate; data represent the mean ± SE.

Table 2. Inhibitory activity of compounds **5c** and **5d** against various PTPs.

PTP	IC_{50} [μM][a] **5c**	**5d**
PTPβ	0.06 ± 0.02	0.1 ± 0.01
LAR	1.7 ± 0.2	1.9 ± 0.5
YopH	1.0 ± 0.04	0.5 ± 0.03
IF1	5.8 ± 0.1	0.5 ± 0.03
IF2	14.3 ± 1.4	27.6 ± 2.9

[a] IC_{50} values were determined by regression analyses and are expressed as the mean ± SE of three replicates.

could be significantly involved in the enhanced affinity of these inhibitors toward PTP1B.

To verify this hypothesis, compounds **2c** and **5d**, which exhibited the highest affinity toward PTP1B, and a less active analogue **2g**, which contains a *m*-methylbenzoic acid residue on its 5-arylidene moiety, were selected for our docking studies. PDB structures 1G7G[29] and 1XBO[26] were selected as templates for human PTP1B. Multiple and varied poses were obtained for our compounds, with different preferences for the two protein templates. The molecules in which the distance between the two carboxylic groups is greater (**2c** and **5d**) showed some interactions with Arg24 and Arg254 in the secondary phosphate site, although the carboxylic acid group on the 5-arylidene moiety was usually not located between them. Interactions with a third acid-group binding location at Lys41 and Arg47 were also frequently detected. Moreover, because of the similar chemical environment around the two carboxylic acids, either carboxylic group can occupy the catalytic phosphate binding site, leading to the possibility of flipped binding modes (Figure 3). Different though they may be, most of the obtained poses look reasonable. In fact, experimental evidence exist for both types of interactions of a second acidic ligand residue: either with the secondary aryl phosphate binding site[21,30,31] or with the third acidic-group binding site.[29,32] The highest-ranked binding pose obtained for **2g** in 1G7G shows that it is only through a strong bend in the molecule that the interactions with Arg24 and Arg254 can be made (Figure 3).

In summary, our optimization efforts led to the identification of new benzoic acids that are active as inhibitors of both human PTP1B and LMW-PTP, by exhibiting improved activity with respect to their parent analogues. Compound **5d** proved to be the most potent PTP1B/LMW-PTP inhibitor, with sub-micromolar IC_{50} values against both human PTP1B and IF1. Molecular docking experiments into the PTP1B active site highlighted that these derivatives can interact with amino acid residues critically involved in the affinity and selectivity of inhibitors toward this enzyme, such as Arg24, Arg254, and Arg47, which is an important result for the future design of new analogues.

Experimental Section

Experimental details including synthesis and workup procedures and conditions, reagent grades, instrument details, molecular modeling procedures, protein preparation and purification, enzyme assays, as well as kinetic analyses are available in the Supporting Information.

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Keywords: benzoic acids · drug design · enzymes · inhibitors · protein tyrosine phosphatases

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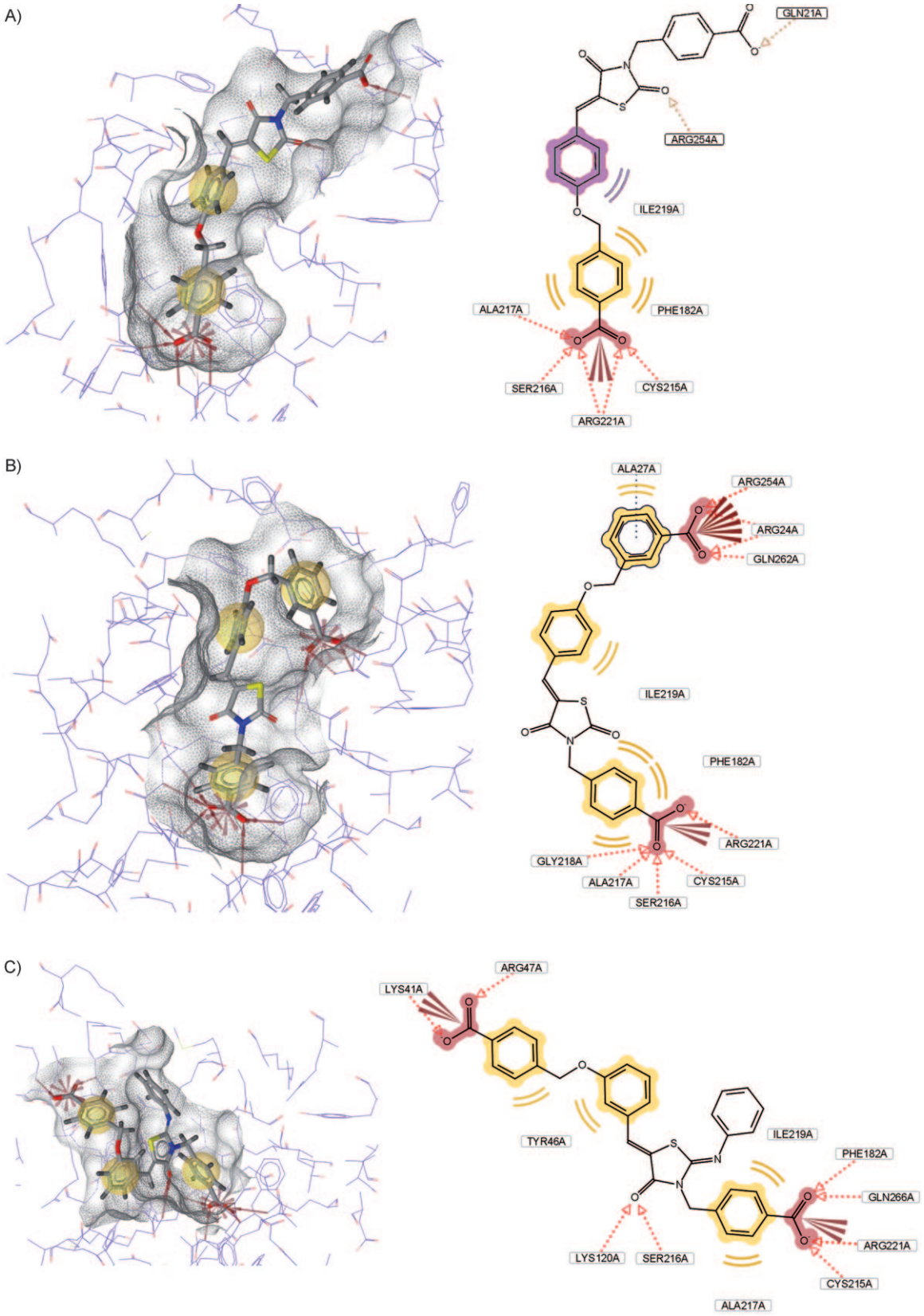

Figure 3. Highest scored docking poses received for A) **2c** (docked into PDB 1XBO),[26] B) **2g** (PDB 1G7G),[29] and C) **5d** (1G7G). Left: 3D representation, right: 2D representation. Pharmacophoric interactions were automatically detected by LigandScout.[33] Yellow spheres: hydrophobic feature, red arrows: hydrogen bond acceptor, red stars: negative ionizable feature. All 3D poses are presented from the same point of view, but the representation of **5d** is shifted toward the left.

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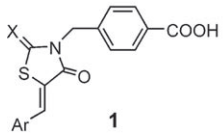

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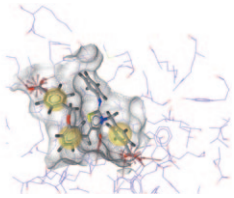

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Structure-Based Optimization of Benzoic Acids as Inhibitors of Protein Tyrosine Phosphatase 1B and Low Molecular Weight Protein Tyrosine Phosphatase

We have optimized previously discovered benzoic acids **1**, which are active as inhibitors of PTP1B and LMW-PTP, two protein tyrosine phosphatases that have emerged as attractive targets for the development of novel therapeutic agents for the treatment of diabetes, obesity, and cancer. Our efforts led to the identification of new and more potent analogues with appreciable selectivity toward human PTP1B and the IF1 isoform of human LMW-PTP.

